INTRAMOLECULAR HETEROGENEITY OF IgM ANTIBODIES

BY

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"Take instruction, and not silver,
And knowledge rather than choicest gold.
For wisdom is better than jewels;
And all desirable things can not compare
with her."

Proverbs 8:10-11 (NAS)
The Bible

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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A hybridoma line (14PAF) secreting 19S IgM antibodies reactive with the DNP moiety was derived from a fusion of DNP-primed murine spleen cells with the myeloma line P3-X63-Ag-8. The failure to demonstrate ten binding sites per pentameric molecule (six were measured) was attributable to the presence of two different light (L) chains. Analysis of the reductive subunits indicated the presence of two types. One type (designated active) contained an average of two homogeneous ligand binding sites per subunit and did not contain L chains normally found in MOPC-21, the myeloma protein secreted by the P3 myeloma line. The other type of subunit (designated inactive) did not contain any DNP binding sites and contained only MOPC-21 L chains. Recombination studies with μ and L chains from each type of reductive subunit indicated that the noncovalent assembly of the 2μ-2L chain subunits was restricted by the L chains

in such a way that the recombinant subunits were homogeneous in terms of L chains. Equilibrium dialysis studies with active homogeneous recombinant subunits indicated the presence of but one ligand binding site per 2μ-2L chain subunit.

Two additional murine anti-DNP IgM hybridoma antibodies were derived using the non-immunoglobulin producing myeloma lines P3-X63-Ag8.653 and SP2/0-Ag-14. These IgM antibodies, NP3-17 C1-20 and SP2/0 I-64 C1-12, displayed an average of five high affinity binding sites for the DNP moiety. Reductive subunits of each of these proteins absorbed to and were hapten eluted from an affinity column. These subunits, when examined by equilibrium dialysis, each contained an average of one high affinity binding site. Structural analysis of these molecules indicated each to be homogeneous. Results obtained from trypsin hydrolysis of each molecule as well as studies performed with reductive halfmers (H-L) indicated that differences in conformation of the binding sites may account for the observed binding heterogeneity.

The results of the chain recombination studies with 14PAF closely mimic the natural situation that occurs with NP3-17 C1-20 and SP2/0 I-64 C1-12. These findings strongly implicate the mechanism of assembly as a possible factor in the generation of conformational differences that could account for the intramolecular heterogeneity of ligand binding seen with certain IgM molecules.

CHAPTER I
BACKGROUND

The structure and physiochemical properties of mammalian IgM immunoglobulins have been elaborated in considerable detail largely through the study of homogeneous IgM myeloma proteins. These studies demonstrate that secreted mammalian IgM antibodies are pentameric molecules containing ten heavy-light chain pairs and thus, by analogy with IgG antibodies, should contain ten equivalent ligand binding sites per molecule (reviewed 1). Although several studies employing structurally homogeneous IgM monoclonal proteins have demonstrated the presence of ten homogeneous, relatively low affinity binding sites per molecule (2,3), numerous other studies with conventional IgM antibodies have indicated considerable heterogeneity of ligand binding with valences of less than ten. In several instances valences of less than ten were attributable to steric factors related to antigen size. For example, in one study the measured valences of IgM antibodies to dextran progressively decreased as the size of the poly-glucosan ligand was increased (4). Similarly in another study a human IgM myeloma protein which bound human IgG was observed to have an effective valence of five per intact pentamer (one per subunit) whereas each of the ten Fabμ fragments contained an active binding site (5). In other cases,

haptens which are not likely to impose steric limitations on the antibody combining sites were employed. In this latter context it is important to point out that an average of five high and five low affinity binding sites has frequently been observed in a variety of species (6,7,8,9). One study utilizing an unusual IgM subunit containing only a single heavy-light chain (10) demonstrated that 50% of these subunits were retained by an immunoadsorbant column previously used to isolate this heterogeneous antibody from serum. Since it was also shown that greater than 90% of the 7S subunits of this IgM antibody were retained by an immunoadsorbant, the hypothesis was suggested that heterogeneity must exist within individual 7S subunits. However due to the fact that the IgM was not homogeneous, critics explained the data by the presence of different populations of high and low affinity IgM molecules, half of which lack sufficient affinity as heavy-light chain pairs to be retained by an immunoadsorbant.

In terms of resolving this issue there would seem to be two different, but not necessarily exclusive, possibilities. The first is that ligand binding heterogeneity is a consequence of differences between antibodies in a population, i.e., intermolecular differences due presumably to differences in antibody primary structure. The second possibility is that ligand binding heterogeneity reflects differences within individual antibody molecules, i.e., intramolecular differences. While the first possibility

is likely the case in some circumstances wherein obviously structurally heterogeneous antibodies are employed, the frequent finding of an average of half high and half low ($\sim$ 1% as high as those with high affinity) affinity sites suggests consideration of the second possibility. Furthermore, one attempt at separating the two populations of binding sites suggested that both were on the same IgM molecule (11), hence adding credence to the possibility of intramolecular heterogeneity.

The solution to this question has, to a large part, been elusive due to difficulties in obtaining sufficient amounts of structurally homogeneous IgM antibodies. A potential approach to this question was offered by the observations that the physicochemical properties of immunoglobulins from lower vertebrates indicate that such animals may be restricted to but one immunoglobulin isotype analogous to IgM (reviewed, 12,13). Furthermore, studies using sharks indicated that certain antigens, especially A-variant streptococci, can elicit the production of very large amounts of relatively homogeneous 19S antibodies (up to 10 mg/ml serum) (14,15). Although it has not been possible to isolate a suitable hapten for studying affinities and valences with this antigen (16), the finding in sharks of reasonably good 19S antibody responses to the capsular polysaccharide of pneumococcal cells and to the DNP hapten covalently coupled to streptococcal cells provided sufficient material for limited studies. The results of these studies with shark IgM antibodies are summarized below.

Shark 19S antibodies to the Type III (S_3) pneumococcal capsular polysaccharide were isolated from immune sera by affinity chromatography and subjected to equilibrium dialysis using the S_3 hexasaccharide hapten. The results demonstrated several important points: (1) The IgM antibodies contained an average of ten combining sites per molecule, (2) in all cases, the antibodies showed marked heterogeneity of affinities, (3) the antibodies were all of low average affinities and (4) there was no increase in the average affinity of the antibodies isolated from any single animal for periods up to twelve months after initial immunization. In order to determine if a single IgM molecule contained ten equivalent combining sites, the antibodies isolated from several animals were fractionated by liquid isoelectric focusing. Equilibrium dialysis experiments using focused fractions showed the presence of ten functionally identical combining sites per 19S molecule. As a proof of the structural homogeneity of focused fractions, antibodies were mildly reduced, separated into H and L chains, recombined to 7S subunits (2H-2L chains), and tested for combining sites by equilibrium dialysis. The results indicated that these 7S recombinants of focused antibody fractions each contained two binding sites identical to those of the intact antibody whereas heterogeneous (unfocused) recombinants or isolated H and L chains failed to show any binding activity. The conclusion from this study is that the heterogeneity of ligand binding exhibited

by nurse shark 19S antibodies to the capsular polysaccharide of the Type III pneumococcus can be attributed to intermolecular heterogeneity most likely at the primary structural level (17).

In contrast to the results obtained in sharks with the pneumococcal antigen, those obtained with antibodies to the DNP moiety dictate a quite different conclusion (18). Equilibrium dialysis studies using the hapten DNP-ε-aminocaproate with affinity purified nurse shark 19S antibodies to DNP demonstrated several important points: (1) The 19S antibodies exhibited heterogeneity of ligand binding with an average of five high and five low affinity sites per molecule, (2) the affinities of the low affinity sites were approximately 1% of those of the high affinity sites, and (3) no evidence for increased affinities was seen for up to twenty-one months of immunization. To study the basis for the ligand binding heterogeneity of the shark 19S antibodies to DNP, several different approaches were employed. The results of studies with subunits and proteolytic fragments showed that neither steric hindrance nor allosteric effects could account for the observed heterogeneity, i.e., the two forms of binding sites were on separable Fabμ fragments. In fact these results strongly suggested that both types of combining sites were present within individual reductive subunits. Preparative liquid isoelectric focusing was used in an attempt to separate structurally homogeneous 19S antibodies. The results from equilibrium

dialysis studies indicated that each of 16 different focused preparations contained an average of five high and five low affinity sites. These results seemingly argue strongly against the possibility that the observed heterogeneity was due to intermolecular heterogeneity. It seems highly improbable that each of sixteen different isoelectrically focused anti-DNP preparations would fortuitously be composed of equimolar mixtures of 19S molecules, half of which have ten equivalent ligand binding sites approximately 100 times higher in affinity than those of the remaining low affinity population. It would seem more likely that the isoelectric focusing technique actually separated structurally homogeneous antibodies. This interpretation is supported by the results of recombination studies. Heavy and light chains from focused molecules yielded 7S recombinants with high and low affinity sites, albeit in low yields, indistinguishable from their putative counterparts on the intact molecule. Since heterogeneous recombinants resulted in binding sites of only low affinities, it seems likely the focused preparations were structurally homogeneous and hence the observed ligand binding heterogeneity must be an intramolecular phenomenon (19).

The presence of binding sites of two different affinities on a single antibody molecule can be explained by primary structural and/or conformational differences between the two types of sites. Although the presence of amino acid sequence heterogeneity in the heavy and/or light chains of a

single IgM molecule seems unlikely, the lack of amino acid sequence data on the shark IgM antibodies makes it impossible to a priori rule out this explanation. Despite this unclarified point, evidence from another experimental approach seems to favor the latter possible explanation mentioned above. Shark 19S antibodies to the DNP moiety (exhibiting five high and five low affinity sites) and to the S_3 polysaccharide (exhibiting ten low affinity sites) were each treated with 5 M guanidine-HCl and studied by equilibrium dialysis with the appropriate hapten after removal of the denaturing solvent. The results showed this treatment had no effect on the number or affinity of sites on the antibodies to the S_3 polysaccharide but had a considerable effect on the antibodies to DNP. In fact the data obtained with these latter antibodies indicated linear Scatchard plots that readily extrapolated to a valence of ten low affinity sites. Thus it appears that the guanidine-HCl treatment converted the five high affinity sites to low affinity ones. This was presumably accomplished by causing conformational changes in or near the high affinity sites. Unfortunately sufficient amounts of shark antibodies were not available to perform detailed studies of the conformational relationships between Fabμ fragments containing high or low affinity sites.

In light of the suggestion that the intramolecular heterogeneity of ligand binding by some shark (and perhaps other species) IgM antibodies to the DNP moiety may result

from intramolecular conformational differences, it seems appropriate to speculate on possible mechanisms responsible for these putative differences. One possible explanation is that IgM molecules may be assembled intracellularly by two different mechanisms depending upon the cell involved. Since the data discussed above suggest these differences are in fact intrasubunit differences, it is conceptually sound to suggest that those lymphocytes secreting IgM antibodies with ten homogeneous sites may do so by assembling the subunits (2H-2L) from halfmers (H-L) as is the case for a relatively limited number of IgM myeloma proteins studied (20,21). In this case it would be predicted that the subunits should exhibit symmetry of ligand binding. On the other hand, those cells secreting IgM antibodies with half high and half low affinity sites may do so by assembling the subunits in a different manner, i.e., either H-H→H-H-L→L-H-H-L or H-L→H-H-L→L-H-H-L. If these latter modes of assembly exist, as is the case with certain other immunoglobulin isotypes (22), it is conceivable (although admittedly antidogma) that the formation of the first H-L chain pair may somehow influence the conformation of the second pair so as to result in a different affinity site. A priori, one cannot tell which (high or low affinity) site would be formed first.

Since the suggestion of intramolecular conformational differences may seem to some to reflect too much "antidogma," it would be appropriate here to briefly consider this issue.

There are several reports in the literature which document the existence of small but detectable conformational differences between Fab fragments derived from specific antibodies and immunoglobulins (23,24). Admittedly these proteins were heterogeneous (intermolecular) in primary structure and hence the existence of such conformational differences may not be surprising. Furthermore, while the relatively recent surge of X-ray crystallographic data has indicated considerable 3-dimensional similarities between several homogeneous Fab fragments, there are also indications of slight differences (reviewed 25). Perhaps the most important observations that could be cited in support of the possible existence of intramolecular conformational heterogeneity are those of Edmundson and colleagues (26) with the myeloma protein and dimer Bence-Jones protein of patient Mcg. These data indicate rather conclusively that different conformations of a polypeptide chain (L chains in their study) can be derived from the same amino acid sequence depending upon the other chain to which the peptide is paired. Similar precedents have been reported for the crystalline structures of dimeric insulin (27) and chymotrypsin (28). Thus it does not seem absurd at this point to suggest that such conformational differences could exist and be functionally important within structurally homogeneous IgM molecules. Finally, since part of our rationale for suggesting the role of intramolecular conformational differences to explain the apparent intramolecular heterogeneity

of ligand binding of some IgM antibodies was based upon the results obtained with guanidine-HCl treated antibodies, the results of Richards and coworkers with mouse IgA myeloma protein 460 become pertinent (29). Their data indicated that this protein, possessing two ligand binding specificities, could be rendered unreactive with one ligand (DNP) by the guanidine-HCl treatment whereas the affinity for the other (menadione) was unaffected. Presumably this partial loss in function resulted from an intracombining site conformational change. It is unknown if such a change could be detected by the methodology available.

CHAPTER II
INTRASUBUNIT HOMOGENEITY IN HETEROGENEOUS IgM ANTIBODIES TO THE DNP MOIETY DERIVED FROM A MURINE HYBRIDOMA CELL LINE

Introduction

In light of the immunologic importance of IgM it becomes imperative to develop approaches for obtaining sufficient amounts of structurally homogeneous mammalian IgM antibodies with specificities for defined ligands in order to clearly resolve the question of intramolecular heterogeneity. The approach that seemed most promising and was undertaken involved cell fusions (hybridomas). Chapter II describes the initial results obtained with one murine hybridoma line secreting 19S IgM antibodies reactive with the DNP moiety. Although the secreted IgM product was a structurally heterogeneous molecule (due to the presence of two different light chains) it appeared to be assembled in a random fashion from two pools of homogeneous subunits. Furthermore *in vitro* recombination studies with the component polypeptide chains revealed the surprising findings that a) the subunit homogeneity was L chain directed and b) each recombinant subunit exhibited but one active site.

Materials and Methods

Hybridomas

The fusion techniques described by Galfre' et al. (30) and Gerhard et al. (31) were employed with some modifications. Briefly 1 x 10^8 BALB/c spleen cells (mice injected 3 days previously with 50 µg 2,4-dinitrophenyl-ficoll) and 1 x 10^7 myeloma cells (P3-X63-Ag8; derived by Cotton and Milstein) (32) were mixed and centrifuged at 300g for 8 minutes. Cells were resuspended for fusion in 1 ml of a 50% solution (v/v) of polyethylene glycol (PEG-1000, J.T. Baker Chemical Co.) in Dulbecco's Minimal Essential Medium (DMEM, Gibco). This suspension was further diluted at a rate of 6 ml/min with DMEM over a period of 5 minutes. The cells were washed and resuspended in 35 ml of the selective medium which contains hypoxanthine, aminopterin, and thymidine (33). The cells were distributed in 100 µl aliquots into 96 well microtiter plates (Falcon Microtest II) and incubated at 37°C in a humidified 5% CO_2-95% air mixture. After substantial growth (∿ 10-12 days) supernatants were screened for IgM antibodies to the DNP moiety using a radioimmunoassay (34). Cultures positive for anti-DNP antibody were cloned by limiting dilution in soft agarose (35). Individual positive clones (∿ 5 x 10^6 cells) were injected into BALB/c mice primed with pristane to obtain ascitic tumors. One such clone, designated 14PAF, was selected for the studies reported here.

Immunochemical Procedures

Mouse antibodies to the DNP moiety were purified from ascitic fluid by affinity chromatography, freed of hapten, and examined by equilibrium dialysis against ^{3}H-DNP-ε-aminocaproate as described previously (18). For calculating protein concentrations of the pentameric IgM antibodies, a molecular weight of 900,000 daltons and an extinction coefficient ($E^{1\%}_{280nm\ 1cm}$) of 11.9 were assumed.

In one experiment 14PAF was subjected to 5.0 M guanidine-HCl for one hour at room temperature. The guanidine-HCl was removed by dialysis against Tris buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4). These guanidine-HCl treated antibodies were subjected to equilibrium dialysis as previously described.

Mildly reduced 7S subunits of protein 14PAF were prepared by subjecting purified 19S material to reduction with 0.1 M 2-mercaptoethanol in 0.5 M Tris-HCl, pH 8.0, for one hour at 22°C followed by alkylation with 0.15 iodoacetamide for one hour on ice. Gel filtration under nondenaturing conditions (Sephadex G-200 equilibrated with 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4) indicated that > 95% of such reduced and alkylated 7S material eluted in a volume expected to contain ∿180,000 dalton proteins; analysis under denaturing conditions without additional reduction indicated that >90% of the 7S subunits dissociated into equimolar H and L chains. A small percentage of halfmer (H-L) molecules was also detected. Mildly reduced and alkylated

H and L chains and halfmers were obtained by gel filtering the 7S subunits on Agarose A5M columns equilibrated with 5 M guanidine-HCl containing 0.01 M iodoacetamide.

Recombinant molecules were prepared from mildly reduced and alkylated H and L chains as described previously (17). Briefly, the desired amounts of separated H and L chains were mixed in 5 M guanidine-HCl containing 0.01 M iodoacetamide and concentrated by positive pressure dialysis to ∿ 5 mg/ml while dialysing against Tris buffer, pH 7.4. These recombinants were gel filtered under nondenaturing conditions and >90% of the UV absorbing material eluted in a volume expected to contain 180,000 dalton proteins; electrophoresis of these recombinants in SDS polyacrylamide gels indicated the presence of equimolar H and L chains (see Figure 5 for example). Alkaline-urea gel electrophoresis of extensively reduced L chains was performed by the method of Reisfield and Small (36). SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (37). Amino terminal sequence studies were performed by Edman degradation using an automated Beckman sequenator. PTH derivatives were identified by high pressure liquid chromatography (38).

Results

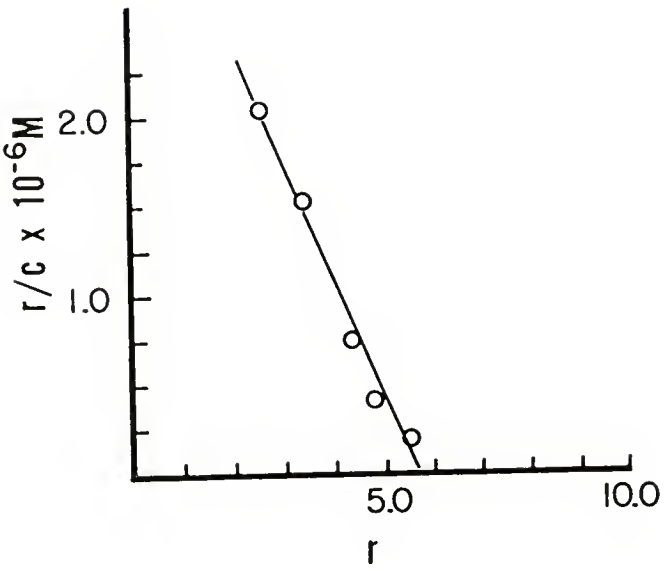
The hybridoma line (14PAF) used here readily proliferated as ascitic tumors in pristane-primed BALB/c mice and yields of 10-20 ml ascitic fluid were obtained from individual mice. Affinity chromatography of these ascitic fluids

on TNP-lys-sepharose yielded from 5-10 mg/ml antibody. These isolated antibodies were considered to be exclusively 19S IgM(K) based upon sedimentation velocity measurements, immunodiffusion analysis with commercial antisera and SDS-gel electrophoresis studies (data not shown).

Equilibrium dialysis of the isolated 19S antibody against the hapten 2,4-dinitrophenyl-ε-aminocaproate yielded the Scatchard plot depicted in Figure 1. These data indicate the hybridoma derived IgM antibody contained an average of about six binding sites per molecule with an affinity of $2 \times 10^{6} M^{-1}$. Furthermore, Sips analysis indicated a heterogeneity index of 0.98 which presumably reflected a high degree of homogeneity in the binding constants of the sites being detected. Equilibrium dialysis of the 19S antibody treated with 5.0 M Gn-HCl demonstrated an identical Scatchard plot to the untreated antibody (see Figure 1).

In order to determine if the observation of about six binding sites per molecule was due to some peculiar steric effects, reductive 7S subunits of protein 14PAF were prepared and studied by equilibrium dialysis. As can be seen in Figure 2, these 7S subunits appeared to exhibit a ligand binding pattern identical to that observed with the parent pentameric molecule, i.e., ∿1.2 sites with an affinity of $\sim 2 \times 10^{6} M^{-1}$ per every two H-L chain pairs. These reductive subunits were then subjected to affinity chromatography on DNP-lysine-sepharose. Approximately 55-60% of the material,

Figure 1. Equilibrium dialysis of mouse hybridoma protein 14 PAF with DNP-ε-aminocaproate.


r/c x 10⁻⁶M
2.0
1.0
5.0
10.0
r

designated as active, absorbed and was hapten-eluted from the affinity matrix; the other 40-45%, designated as inactive, did not absorb. Each of these populations of subunits was examined by equilibrium dialysis. As presented in Figure 2, the active subunits exhibited two homogeneous binding sites identical with respect to affinity to those seen in the unfractionated material. The inactive subunits contained no demonstrable binding sites for DNP.

The finding that both the active and inactive subunits could be derived from functional IgM molecules prompted the hypothesis that the secreted pentameric IgM may be assembled randomly from these two populations of subunits. Thus, as an indirect test of this hypothesis, an experiment was undertaken to attempt fractionating the secreted 19S molecules into subpopulations with differing numbers of hapten binding sites. Specifically purified protein 14PAF was reabsorbed to an affinity column and sequentially eluted with increasing (arbitrary) amounts of DNP-OH. The eluted components were freed of DNP-OH, quantified for protein and studied by equilibrium dialysis. The results, depicted in Figure 3, indicate that the initial pentameric IgM antibody population was composed of subpopulations with different numbers of binding sites. While this experiment was not "fine tuned" to the point where it is possible to clearly say that the pentamers were assembled in a random fashion from the two forms of subunits, the results are certainly suggestive.

Figure 2. Equilibrium dialysis with DNP-ε-aminocaproate of unfractionated (●—●), and active (○—○), and inactive (⊗—⊗) 7S subunits from 14PAF.

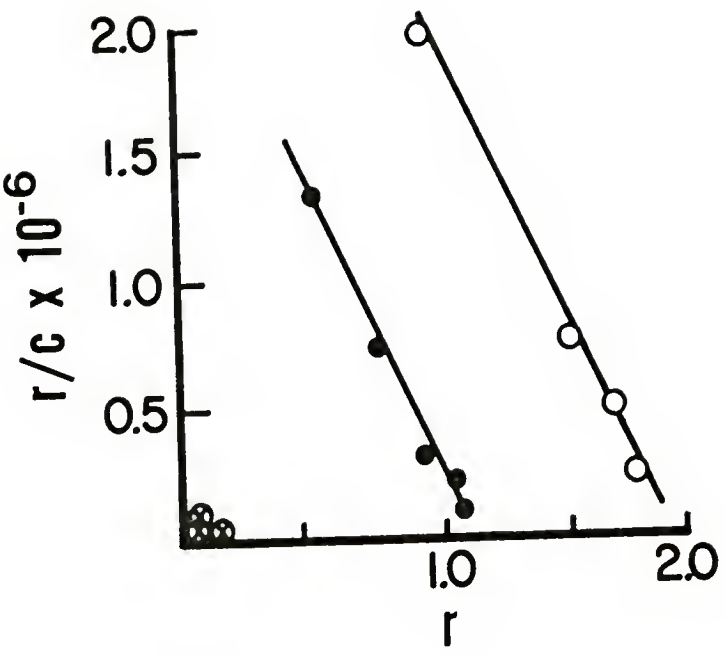
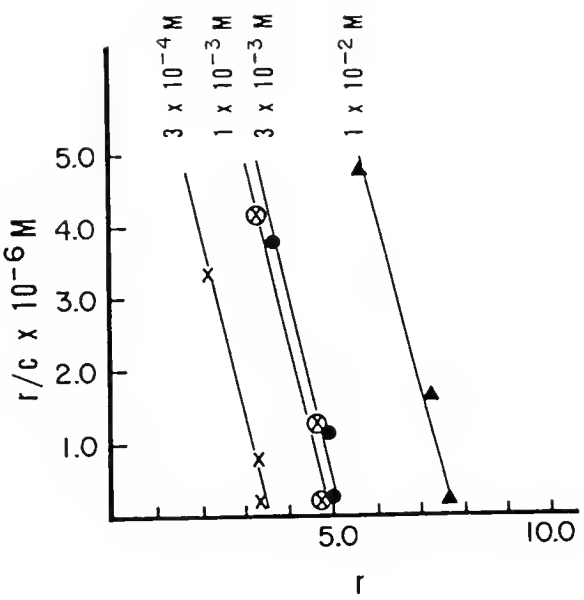

2.0
1.5
1.0
0.5
r/c x 10⁻⁶
1.0
2.0
r

Figure 3. Step-wise elutions of protein 14PAF from DNP-lys-sepharose affinity column using four concentrations of hapten. 18% of the eluted protein was recovered using 3×10^{-4} M hapten; 39% using 1×10^{-3} M; 30% using 3×10^{-3} M, and 13% using 1×10^{-2} M.


3 x 10⁻⁴ M
1 x 10⁻³ M
3 x 10⁻³ M
1 x 10⁻² M
r/c x 10⁻⁶ M
5.0
4.0
3.0
2.0
1.0
5.0
10.0
r

In light of the above finding of two apparently distinct subpopulations of subunits, the possibility of intramolecular heterogeneity at the structural level was considered. The first approach to this question involved subjecting the mildly reduced and alkylated L chains from each subunit to SDS polyacrylamide gel electrophoresis without additional reduction. The banding patterns of these mildly reduced chains were clearly different in that the L chains from the active subunits exhibited a somewhat slower mobility than those from the inactive subunits (see Figure 6 for example); extensive reduction of each of these chains resulted in mobilities expected of $\sim$ 22,000 dalton peptides. The second approach involved subjecting extensively reduced and alkylated light chains isolated from the unfractionated subunits and the two fractionated subunit subpopulations to alkaline-urea gel electrophoresis. The gel patterns of the L chains from the two subpopulations were clearly distinct (Figure 4). These suggestions of L chain differences between the two forms of subunits prompted limited amino acid sequence studies. The results of these studies (Table 1) indicated several important points. Firstly, the L chains from the unfractionated parent molecules exhibited considerable primary structural heterogeneity with two different amino acids being demonstrable at 5 (and possibly 7) different positions in the first 12 residues. Secondly, the L chains from the active and inactive reductive subunits were each homogeneous (through 12 residues) but quite

Figure 4. Alkaline urea polyacrylamide gel electrophoresis of isolated light chains from unfractionated (A), inactive (B), and active (C) 7S subunits.

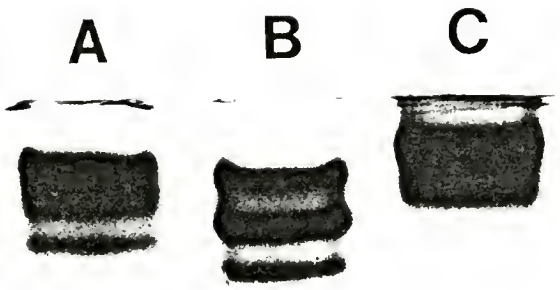

A
B
C

TABLE 1

Amino Terminal Sequences of Light Chains from Unfractionated 19S and Active and Inactive 7S Reductive Subunits from 14 PAF

Position	19S unfractionated	7S subunits Active	7S subunits Inactive	MOPC 21
1	Asp - Asn	Asp	Asn	Asn
2	Val - Ile	Val	Ile	Ile
3	Val	Val	Val	Val
4	Met	Met	Met	Met
5	Thr	Thr	Thr	Thr
6	Gln	Gln	Gln	Gln
7	Thr*	Thr*	Ser	Ser
8	Thr - Pro	Thr	Pro	Pro
9	Leu	Leu	Lys	Lys
10	Ser	Ser	Ser	Ser
11	Leu - Met	Leu	Met	Met
12	Thr*	Thr*	Ser	Ser

The amino terminal sequence of the light chains from MOPC-21 (Svasti and Milstein, 1971) is shown for comparison.

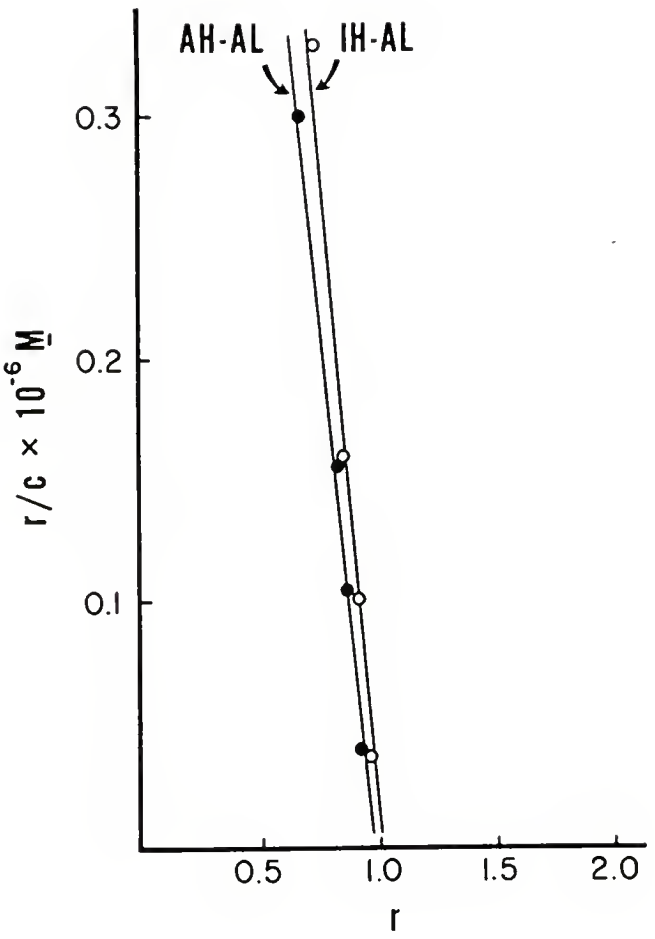
*Ser could be masked

different from each other. A comparison of the amino terminal sequences of the L chains from these two subpopulations of subunits with the L chains from MOPC-21 (the L chain secreted by the myeloma cell line used in the original fusion) indicated that those from the inactive subunits were identical (through 12 residues) to those from MOPC-21. The L chains from the active subunits were quite different. Hence it seems quite likely that the latter L chains were encoded for by the genome of the plasma cell used in the fusion. Therefore the secreted pentameric IgM product of the hybrid cell contained both MOPC-21 and "anti-DNP" L chains.

The μ chains from both the active and inactive subunits from protein 14PAF were blocked at the amino terminus (presumably due to a pyrollidone carboxylic acid) and thus were not amenable to automated sequence analysis. In an attempt to ascertain if these μ chains were functionally identical, recombinant non-covalent subunits were produced between all possible equimolar combinations of separated mildly reduced and alkylated μ and L chains derived from both types of subunits. Each of these recombinants was gel filtered and the ∿ 180,000 dalton material (representing >90% of the total) subjected to affinity chromatography on DNP-lys-sepharose. Greater than 95% of the recombinants formed with L chains from active subunits and μ chains from either active or inactive subunits were observed to absorb to (and to subsequently hapten elute from) the affinity

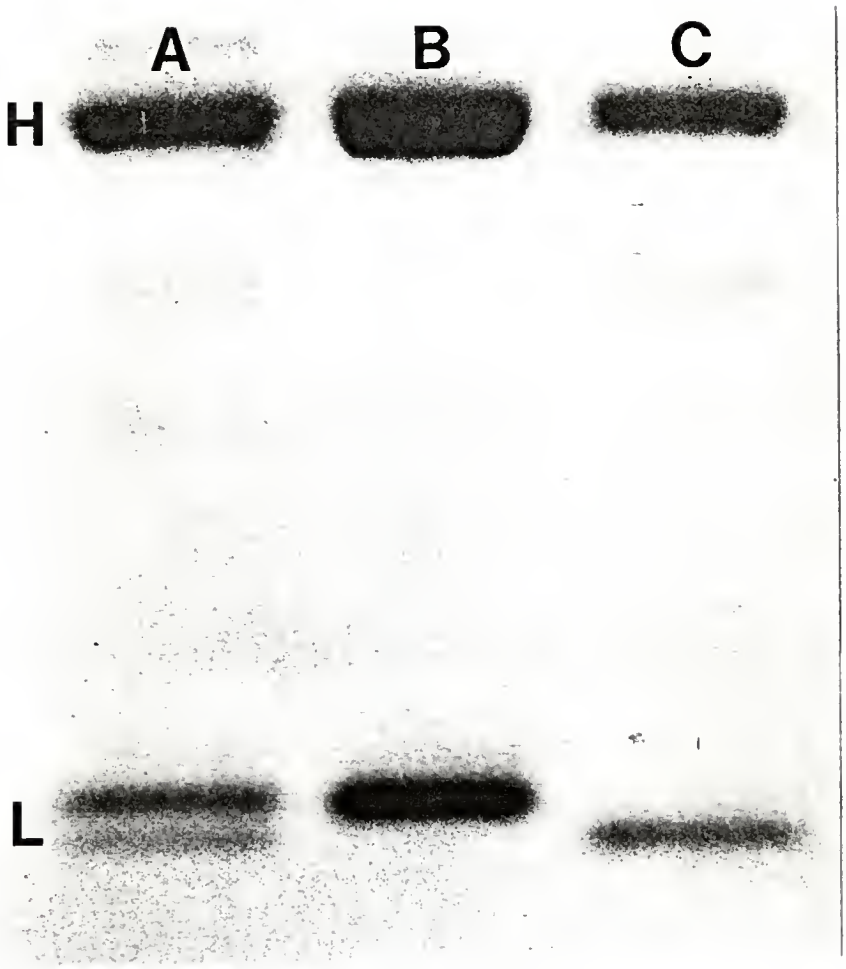
columns; less than 5% of either of the recombinants formed with L chains from inactive subunits absorbed to the columns. Equilibrium dialysis with these latter inactive recombinants indicated no detectable combining sites for the DNP group; similar studies had indicated that the isolated μ or L chains bound no detectable hapten (data not shown). On the other hand, the active recombinants recovered by hapten elution from immunoadsorbant columns were each observed, in duplicate experiments, to have an average of about one combining site with an affinity identical to that of the parent molecule (Figure 5). Similarly a single study was performed utilizing halfmers (covalently linked H-L's) from active subunits obtained from the leading edge of the H chain peak on the 5 M guanidine Agarose A5M column. After removal of the guanidine by dialysis against Tris buffer, these subunits (∿ 180,000 dalton material by gel filtration) displayed an average of one combining site (identical to Figure 5). To further prove the functional identity of the μ chains present in the original 19S molecules, mildly reduced and alkylated μ chains from either the active or inactive reductive subunits were recombined with an equimolar amount of mildly reduced and alkylated L chains derived from the original molecule (i.e., ∿60% "anti-DNP" and ∿ 40% MOPC-21 L chains). Each of these recombinants was then gel filtered on Sephadex G-200 and the 2H-2L chain recombinants (representing >90% of the material) were subjected to affinity chromatography on DNP-lys-sepharose columns;

Figure 5. Equilibrium dialysis with DNP-ε-aminocaproate of 14PAF recombinants using either heavy chains from active subunits (AH) or inactive subunits (IH) and light chains from active subunits (AL). Recombinants made using either heavy chain with light chains from inactive subunits showed no detectable binding.


AH-AL
IH-AL
0.3
0.2
0.1
r/c × 10⁻⁶ M
0.5
1.0
1.5
2.0
r

approximately 40% of the applied material in each case failed to absorb to the column whereas the remainder absorbed and was recovered by hapten elution. The findings from this experiment were a) the recombinant subunits which absorbed to the affinity column contained an average of one binding site identical in affinity to that seen in the previous active recombinants (Figure 5) and the original molecules, and b) the active recombinants contained only L chains with an SDS polyacrylamide gel electrophoretic mobility characteristic of the "anti-DNP" L chains whereas the inactive recombinants appeared to contain only MOPC-21 L chains (Figure 6). Soft laser scans of these stained gels clearly indicated each of the recombinants to be composed of equimolar μ and L chains. In a further attempt to ascertain if 14PAF μ chains preferentially form recombinants with either of the two forms of L chains, mildly reduced and alkylated chains from active reductive subunits were mixed with a two-fold molar excess of 14PAF L chains (consisting of a 60:40 ratio of "anti-DNP" and MOPC-21 L chains). The resultant mixture was, without subsequent gel filtration, then subjected to affinity chromatography on a DNP-lys-sepharose column; approximately 52% of the material failed to absorb to the column whereas the remaining 48% absorbed and was recovered by hapten elution. Again, as in the above case where equimolar μ and L chains were mixed, the active recombinants contained equimolar μ chains and "anti-DNP" L chains when examined by SDS gel electrophoresis.

Figure 6. SDS-PAGE of recombinants formed using heavy chains from either active or inactive subunits combined with an equimolar amount of light chains consisting of 60% from active subunits and 40% from inactive subunits. Lane A represents unfractionated recombinants; Lane B represents recombinants which absorbed to and were subsequently hapten eluted from a DNP-lys-sepharose affinity matrix; and Lane C represents recombinants which did not absorb to the affinity column.


A
B
C
H
L

The inactive fraction from this experiment contained μ chains and a greater than three-fold molar excess of L chains; these L chains were present in a 55:45 ratio of MOPC-21 to "anti-DNP" L chains. The implications of these results are considered below.

Discussion

The original purpose of the work undertaken here was to utilize murine hybridomas as sources of homogeneous IgM antibodies to test the hypothesis that certain IgM antibodies can exhibit intramolecular heterogeneity of ligand binding that is not attributable to primary structural differences. Although the presence of two different L chains in protein 14PAF precludes its utility in this regard, the studies reported here did reveal several novel and potentially important aspects of IgM structure and function. It was not surprising, based upon the observation of "mixed" molecules secreted by other hybridoma cell lines derived from Ig secreting myeloma cells (39), that protein 14PAF contained two different L chains. It was surprising, however, that protein 14PAF was seemingly randomly assembled from 2μ-2L chain subunits that were homogeneous in terms of L chains. This finding suggests that each type of subunit may be assembled from μ-L chain (or perhaps μ-μ-L chain) (40) intermediates which can only associate with homologous μ-L (or L) chain intermediates to form 2μ-2L chain subunits. The possibility that this putative L chain restriction at this level is due to the presence of two different μ chains

is seemingly ruled out by the results of the *in vitro* recombination studies. The finding that μ chains from either type of reductive subunit (active or inactive) could combine with "anti-DNP" L chains to form active recombinant subunits certainly indicates a high level of functional similarity in these chains. Similarly, the observation of homogeneous 2μ-2L chain recombinants formed with a mixture of L chains and μ chains from either type of subunit argues strongly that the intrasubunit L chain restriction is attributable to the L chains themselves. In this regard the results obtained from the recombination experiment using a two-fold molar excess of L chains to μ chains are particularly important in that they show rather conclusively that either type of L chain can combine equally well with the μ chains. For example if the μ chains had a preference for recombining with the "anti-DNP" L chains, no μ chains would be expected in the inactive recombinant fraction as they would all be found in the active fraction. Likewise, if the μ chains had shown a preference for the MOPC-21 chains, $\geq$ 85% of the recombination mixture should have been found in the inactive recombinant fraction. As pointed out in the results, 52% of the recombinant mixture was found in the inactive fraction (hypothetically, one would predict 53% if assembly was random for each type L chain). Furthermore, the presence of "anti-DNP" L chains along with μ chains in the inactive mixture demonstrates that the "anti-DNP" L chains were not a limiting factor in

forming recombinants but that the μ chains must have an equal capacity to form inactive recombinants with MOPC-21 L chains or active recombinants with "anti-DNP" L chains. In addition the presence of ∿ 55% MOPC-21 L chains to ∿ 45% "anti-DNP" L chains in the inactive fraction is almost precisely what would have been expected if assembly of the recombinants was random for both L chains (hypothetically, one would predict 57% MOPC-21 L chains and 43% "anti-DNP" L chains). It thus appears that assembly of 14PAF subunits *in vitro* mimics assembly of subunits *in vivo* both with respect to ratios of active to inactive subunits and the homogeneity in terms of L chains. Hence, the conclusion from this study is that the combination of one of the L chains with the μ chain produced by this cell line results in an assembly intermediate that will only associate with a homologous μ-L chain pair to form a subunit. Such restriction may result from different L chain imposed μ chain conformational differences; future work is required to determine if such putative μ chain conformational differences are caused by the seemingly large conformational differences (as manifested by the differences in SDS gel electrophoretic mobilities) between mildly reduced and alkylated MOPC-21 and protein 14PAF "anti-DNP" L chains.

A final point of interest, and potential importance, from these initial studies with protein 14PAF was the failure to achieve the anticipated number of combining sites in the active recombinants. The 2μ-2L chain *in vitro*

recombinants formed with μ and L chains (or halfmers) from active 2μ-2L chain reductive subunits should have exhibited two ligand binding sites if the *in vitro* recombination process worked perfectly, i.e., such as seemingly was the case during intracellular assembly (see Figure 2). On the other hand, due to the technical intricacies of such experimental manipulations, a loss of (or failure to recover) some of the active sites would not be unexpected. Thus, for the sake of discussion, if 50% of the sites were lost (as suggested by the data in Figure 5) it would be expected that such losses might be random and hence about 25% of the recombinants should have two sites, 50% have one site and 25% have no active sites. In such a case, only 75% of the 2μ-2L chain recombinants would be expected to absorb to an affinity matrix; these absorbed and hapten eluted recombinants should exhibit an average of 1.3 sites per molecule. The results obtained with 2μ-2L chain recombinants formed with protein 14PAF "anti-DNP" L chains and either of the two μ chains (from active or inactive reductive subunits) clearly showed that >95% of the recombinants absorbed to a DNP affinity column and therefore had at least one active site. Furthermore equilibrium dialysis with these active recombinants indicated an average valence of only one. Hence the conclusion from these results must be that *each* 2μ-2L chain recombinant had but *one* active site for ligand binding. The reason for this rather striking result is unknown but it would seem appropriate to speculate

that it may be attributable to intrasubunit conformational differences arising during the *in vitro* assembly; perhaps one mildly reduced and alkylated μ-L chain somehow influenced the conformation of the other pair to result in a 2μ-2L chain recombinant with but one active site for the DNP moiety. Such conformational differences, if demonstrable in the *in vitro* recombinants of protein 14PAF, would certainly justify future studies regarding this possible explanation for heterogeneity of ligand binding by other IgM antibodies.

CHAPTER III
INTRAMOLECULAR HETEROGENEITY OF TWO IgM ANTIBODIES TO THE DNP MOIETY DERIVED FROM MURINE HYBRIDOMA CELL LINES

Introduction

As previously discussed the results obtained by several different laboratories using IgM antibodies from a wide variety of sources indicated that some, but not all, of these antibodies appeared to exhibit intramolecular heterogeneity of ligand binding. As mentioned, these earlier studies were limited by the relatively small amounts and the structural heterogeneity of the IgM molecules available. In order to clearly resolve this question of intramolecular heterogeneity it has become imperative to develop approaches for obtaining sufficient amounts of structurally homogeneous mammalian IgM antibodies with specificities for defined ligands. Chapter II describes the initial attempt at obtaining a homogeneous anti-DNP IgM antibody using hybridoma technology. The study of the molecule obtained, designated 14PAF, resulted in some rather interesting and novel observations which might be pertinent to the question of intramolecular heterogeneity. However, it was felt that this molecule would not, in reality, permit an adequate assessment of the hypothesis regarding intramolecular heterogeneity due to the presence of two different L chains in the secreted pentamer.

This present chapter describes additional cell fusions which were undertaken using two myeloma lines, NP3 (41) and SP2/0 (42), which do not produce immunoglobulin. Two of the cell lines obtained from these fusions have been designated NP3-17 C1-20 and SP2/0 I-64 C1-12. Each of these cell lines grows as ascitic tumors in mice and yields moderate amounts ($\sim$ 1 mg/ml) of 19S IgM antibodies to the DNP moiety. Although apparently structurally homogeneous (as manifested by alkaline urea gel patterns of isolated L chains from both proteins and by limited amino acid sequence analysis of H and L chains from one, NP3-17 C1-20), the hapten binding data obtained for each of these proteins indicate an average of only five high affinity binding sites for the DNP group per 19S molecule. Greater than 95% of the reductive 7S subunits absorbed to and were hapten eluted from a DNP-lys-sepharose affinity column. When examined by equilibrium dialysis, each subunit contained an average of one high affinity binding site. One experiment aimed at defining the molecular basis of this observed binding heterogeneity attempted to determine if asymmetrical carbohydrate attachment to the molecule is in any way involved. Tunicamycin, an antibiotic that prevents glycosylation of glycoproteins, was used in an effort to isolate carbohydrate-free IgM antibodies and to study their binding properties.

Other studies attempting to define the molecular basis of the observed binding heterogeneity involved physically

separating the two types of sites. Mild reduction of each of these IgM molecules yielded predominantly halfmers (H-L) in dissociating buffers. Experiments designed to disrupt the non-covalent associations between opposing halfmers of a 7S subunit, as well as trypsin digestion of both 7S molecules, indicated that conformational differences may exist between the two binding site regions of an individual 7S subunit. A proposed mechanism for this heterogeneity will be discussed.

Materials and Methods

Hybridomas

Cell fusions were performed as described in Chapter II using the myeloma cell lines P3-X63-Ag8.653 (41) and SP2/0-Ag-14 (42). After substantial growth, culture supernatants were screened for IgM antibodies to the DNP moiety using a radioimmunoassay (34) or passive hemagglutination with DNP conjugated sheep red blood cells. Cultures were cloned in soft agarose (35) and individual positive clones were subsequently injected into BALB/c mice primed with pristane to obtain ascitic tumors. Two such clones, designated NP3-17 Cl-20 and SP2/0 I-64 Cl-12, were selected for the studies reported here.

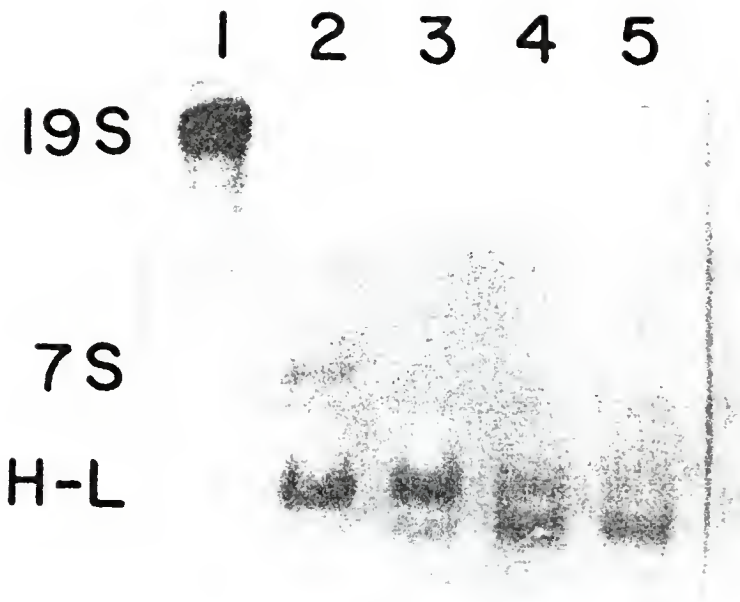
Immunochemical Procedures

IgM antibodies to the DNP moiety were purified from ascitic fluid by affinity chromatography, freed of hapten by Dowex 1X-8 ion exchange chromatography, and examined by equilibrium dialysis against ^{3}H-DNP-ε-aminocaproate as

described previously (18). For calculating protein concentrations of the pentameric IgM antibodies, a molecular weight of 900,000 daltons and an extinction coefficient ($E_{280nm}^{1\%}{}_{1cm}$) of 11.0 were assumed.

Initially, reduced 7S subunits of both proteins were prepared by subjecting purified 19S material (concentrations ranged from 3-10 mg/ml) to reduction with .01 M and .005 M 2-mercaptoethanol for IgM antibodies from NP3-17 C1-20 and SP2/0 I-64 C1-12, respectively. These levels of 2-ME were determined empirically for each molecule as that level of reducing agent which yielded the greatest percentage of subunits containing covalently linked H and L chains or halfmers (covalently linked 2H-2L chain 7S subunits could not be obtained; Figure 7). These reductions were performed in 0.5 M Tris-HCl, pH 8.0 for one hour at 22°C followed by alkylation with 0.15 M iodoacetamide for one hour on ice. Gel filtration under non-denaturing conditions (Sephadex G-200 equilibrated with 0.15 M NaCl, .01 M Tris-HCl, pH 7.4) indicated that ∿ 95% of such reduced and alkylated 7S material eluted in a volume expected to contain ∿ 180,000 dalton proteins; analysis under denaturing conditions without additional reduction indicated the 7S subunits dissociated predominantly into halfmers (H-L molecules). Subsequently it was found that halfmers could be obtained in non-denaturing buffers by mild reduction of dilute protein solutions (.5-1.0 mg/ml) in .5 M Tris-HCl pH 8.5.

Figure 7. Mild reduction profile of 19S IgM from hybridoma SP2/0 I-64 C1-12 using increasing amounts of 2 ME from left to right. Lane 1, .001 M; Lane 2, .005 M; Lane 3, .01 M; Lane 4, .05 M; Lane 5, .1 M. Profiles of IgM from NP3-17 C1-20 were similar with .01 M 2 ME being optimum for the production of halfmers (H-L).


1 2 3 4 5
19S
7S
H-L

Alkaline-urea gel electrophoresis of extensively reduced L chains was performed as in the preceding chapter by the method of Reisfield and Small (36). SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (37).

Amino terminal sequence studies were performed by Edman degradation using an automated Beckman sequenator. PTH derivatives were identified by high pressure liquid chromatography (38).

Tryptic hydrolysis of 7S reductive subunits (5-10 mg/ml) was performed for various time periods following the protocol of Klapper et al. (43). Briefly a 1:100 enzyme (trypsin, 3-X crystallized, Worthington Biochemical Corp.) to substrate ratio was established and the temperature was maintained at 37°C throughout the digestion. Trypsinization was done in .25 M Tris-HCl, pH 8.3, and .01 M $CaCl_2$ was added to the reaction mixture prior to adding trypsin (44). Tryptic digestions were halted by the addition of equimolar amounts of soybean trypsin inhibitor (45). Fab_μ fragments were isolated by gel filtration (G-200 Sephadex equilibrated with 0.15 M NaCl, .01 M Tris-HCl, pH 7.4). Active Fab's were then selected using a DNP-lysine-sepharose immunoadsorbant. Equilibrium dialysis was performed on these Fab's as previously described in this text. A molecular weight of 50,000 and an extinction coefficient of 13.0 were used to calculate Fab concentrations (44).

Attempts were made to dissociate putative active halfmers by disturbing non-covalent interactions between opposing μ chains. Mildly reduced antibody was allowed to absorb to a DNP-lys-sepharose affinity column followed by exposure of the column to chaotropic or ionic dissociating agents. Various concentrations of the following agents were used: guanidine HCl, sodium thiocyanate, acetate buffer (pH 5.0), isopropyl alcohol, NP-40, and Tris-HCl (pH 8.5).

Affinity labeling of reductive subunits from SP2/0 I-64 C1-12 was attempted to irreversibly block the active anti-DNP site on each 7S monomer without affecting the inactive site. A two-fold excess of dinitrofluorobenzene (DNFB) and dinitrobenzenesulfonate (DNBS) to antibody was used. Two milliliters of a 7.2×10^{-6} M concentration of antibody in .1 M borate buffer, pH 8.3 was allowed to react for two hours at room temperature with either DNFB or DNBS at concentrations of 1.44×10^{-5} M. After two hours an equal volume of .1 M Tris-HCl, pH 8.0, was added to each tube to stop the labeling reaction. The antibody was dialyzed against Tris-buffered saline (.15 M NaCl, .01 M Tris-HCl, pH 7.4) overnight. The treated antibody was passed over a DNP-lys-sepharose affinity column and any non-absorbing antibody was measured spectrophotometrically at 280nm. It was hoped that when such treated subunits were placed in 2.0 M guanidine-HCl and allowed to "refold" upon

dialysis against Tris-buffered saline that 50% of the original inactive sites would become active.

Attempts were made to obtain an anti-idiotypic antiserum to IgM from SP2/0 I-64 C1-12. It was hoped that such antiserum might detect idiotypic differences between molecules which could bind hapten and those which could not. Each of three BALB/c mice was initially immunized intraperitoneally with 100 μg of 19S antibody from SP/20 I-64 C1-12 emulsified in complete Freund's adjuvant. Each mouse received three additional intraperitoneal injections of protein without adjuvant over the next two months. One month into the immunization schedule each mouse received 1×10^6 SP2/0.14 myeloma cells in order to produce an ascitic tumor. Three days after the end of the last immunization ascitic fluid was removed and assayed for anti-idiotypic antibodies.

Tunicamycin Experiment

Cells. Six $\times 10^6$ SP2/0 I-64 C1-12 cells were incubated in 10 ml of methionine-free Dulbecco's MEM (with L-glutamine, penicillin and streptomycin) with 15% fetal calf serum. The experiments were run in a humidified atmosphere of 5% CO_2:95% air at 37°C.

Cell labeling. Cells were labeled with ^{35}S-methionine (American Searle, Arlington Heights, IL, 900 Ci/mmole) by the addition of 100 μCi to 10 ml cultures. When tunicamycin (Tm) (Eli Lilly, Co., Indianapolis, IN) was employed, cells

were suspended in the above medium containing 10 μg/ml Tm for one hour before addition of the label. Cells were labeled for eight hours before supernatants were removed for analysis.

Purification of antigen-specific counts. Cells were removed from supernatants by centrifugation at 400g for 15 minutes. Cell-free supernatants were passed over a DNP-lys-sepharose affinity column to purify antigen-specific radioactive molecules. This purified antibody was hapten eluted (.1 M DNP-OH) and subjected to Dowex 1X8 ion exchange chromatography to remove the DNP-OH. It was necessary at this point to spike the radioactive IgM with unlabeled IgM in order to avoid significant loss of radioactivity on the Dowex column. Radioactivity was assessed by adding 10 μl of sample to 2 ml of scintillation cocktail containing 50% (v/v) Triton-X-100.

Gel analysis. Tm-treated and non-treated purified antibodies were subjected to SDS-DATD-acrylamide gel chromatography (46). Gels were dried, overlaid with Kodak X-OMat AR film and stored between intensifying screens for desired exposure times.

Sequential hapten elutions. Purified Tm- or non-Tm-treated antibody was mildly reduced with .1 M 2ME for one hour and alkylated (.15 M iodoacetamide) for one hour on ice. These reductive subunits were allowed to absorb to a small DNP-lys-sepharose column and subjected to step-wise gradient hapten elutions (beginning with 1×10^{-6} M DNP-OH) until all radioactivity was eluted.

Analysis of nonspecific radioactivity from supernatants. Radioactivity from Tm- or non-Tm-treated supernatants was examined for nonspecific IgM by immunoprecipitating supernatant fluid which had been freed of specific antibody by affinity chromatography. Twenty microliters of a rabbit anti-mouse μ chain antiserum was added to .1 ml of anti-DNP free supernatant and incubated for one hour on ice. This was followed by addition of .2 ml of a 50% suspension of Staphylococcus A (Cowan I strain) and incubated for 15 minutes on ice. This mixture was centrifuged for three minutes at 15,000 rpm. Pellets were resuspended in 1% SDS-Tris buffered saline and samples were removed for scintillation counting.

Analysis of cell lysates. Pelleted cells from Tm- or non-Tm-treated cultures were resuspended in 4 ml of .1% NP40 in .04 M Tris-buffered saline with .01 M EDTA, pH 8.3. Cell debris was removed by centrifugation at 500g for twenty minutes. Supernatants were then freed of DNP specific antibodies by affinity chromatography. Both purified antibody and antibody-free supernatants were assayed by the methods described above.

Results

Functional and Structural Characterization of the IgM Antibodies

The hybridoma lines utilized here (NP3-17 C1-20 and SP2/0 I-64 C1-12) grow as ascitic tumors in BALB/c mice and yield moderate amounts (∿ 1 mg/ml) of 19S IgM antibodies to the DNP moiety. Equilibrium dialysis studies using the

hapten DNP-εaminocaproate indicate each to contain an average of five high affinity ($Ka \sim 10^7 M^{-1}$) binding sites (Figure 8). Furthermore, ∿ 95% of the reductive 7S subunits of each of these proteins absorb to and subsequently can be hapten eluted from a DNP-lys-sepharose affinity column. These subunits when examined by equilibrium dialysis again using DNP-ε-aminocaproate indicate each to contain an average of one high affinity binding site. Structural features of these two proteins indicate a considerable degree of homogeneity as might be anticipated using non-producing myeloma cell lines as the parent line for the fusion process. Alkaline-urea gel electrophoresis of the isolated L chains from each protein revealed banding patterns indicating considerably restricted heterogeneity (Figure 9). In addition, limited amino terminal sequence analysis of the H and L chains from the NP3-17 C1-20 protein indicate each to be homogeneous (Table 2). The H and L chains from SP2/0 I-64 C1-12 IgM were not amenable to sequence analysis (presumably due to blocked amino terminii; pyrollidone carboxylic acid).

In experiments designed to measure the binding ability of either of these 19S molecules after treatment with 5.0 M guanidine-HCl for one hour followed by dialysis against Tris buffer, it was found by equilibrium dialysis that the high affinity binding sites were totally destroyed; no low affinity sites could be detected (data not shown).

Figure 8. Equilibrium dialysis of 19S IgM from hybridoma SP2/0 I-64 C1-12 with DNP-ε-aminocaproate. Results for NP3-17 C1-20 were similar.

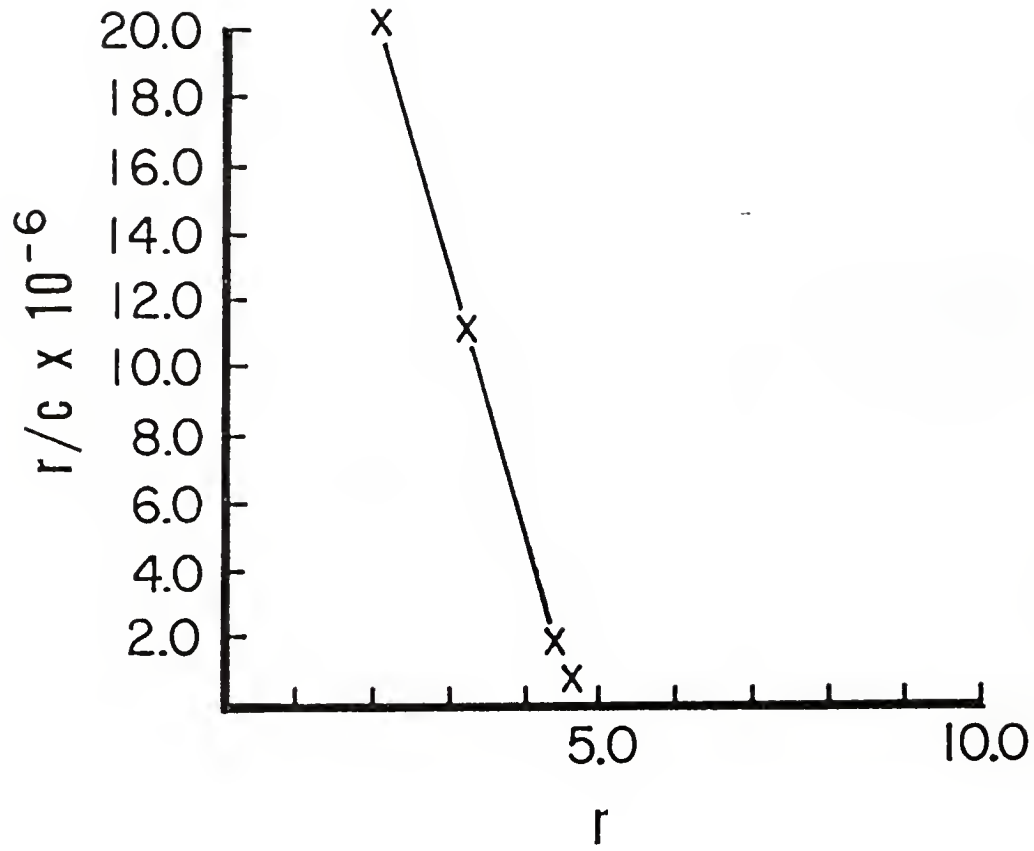

20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
r/c x 10⁻⁶
5.0
10.0
r

Figure 9. Alkaline urea polyacrylamide gel electrophoresis of isolated light chains from (A) SP2/0 I-64 C1-12 and (B) NP3-17 C1-20.


A
B

TABLE 2

Amino Terminal Sequences of Heavy and Light Chains of IgM Antibody to DNP from a Hybridome Line NP3-17 CL-20

RESIDUE	H CHAIN	L CHAIN
1	Glu	Glu
2	Val	Asn
3	Gln	Val
4	Leu	Leu
5	Gln	Thr
6	Gln	Gln
7	Ser	Ser
8	Gln	Pro
9	Pro	Ala
10	Glu	Ile
11	Leu	Met
12	Val	Ser

Separation of Halfmers (H-L)

Mild reduction of either of these IgM molecules resulted in the formation of noncovalently associated halfmers (H-L) in nondissociating buffers. These molecules gel filtered on G-200 Sephadex as would be expected of a 7S, 180,000 dalton protein. However on SDS-PAGE (10% acrylamide) without further reduction these molecules migrated as ∿90,000 dalton proteins, i.e., halfmers. Upon subsequent extensive reduction in SDS the ∿90,000 dalton material dissociated into equimolar ∿70,000 dalton H chains and ∿22,000 dalton L chains. Since these halfmers noncovalently associated in nondissociating buffers it was felt that appropriate denaturing or chaotropic agents might be able to disturb the noncovalent interactions without destroying the binding site of the "good" halfmers, therefore allowing elution and recovery of the "bad" halfmers. Using a "grab bag" approach, various concentrations of the following agents were used: guanidine-HCl, sodium thiocyanate, acetate buffer (pH 5.0), isopropyl alcohol, NP-40, and Tris-HCl (pH 8.5). Initially it was observed that two column volumes of 2.0 M guanidine-HCl would elute 50% of the "7S" antibody from the DNP-lys-sepharose column with the remaining 50% being eluted with .1 M DNP-OH. After dialysis against Tris buffer the guanidine-eluted fraction would subsequently bind to the DNP affinity matrix. Furthermore both the guanidine-eluted and the DNP-eluted material exhibited one binding site per 2H-2L chain

subunit. The major problem in interpreting this experiment was the observation that all of the bound antibody could be eluted if more than two column volumes of 2.0 M guanidine-HCl was used. Therefore there are two distinctly different interpretations of the data: 1) It is possible that the two column volumes of guanidine-HCl were eluting preferentially the "bad" halfmers from the affinity column and that any more than two volumes began to denature and elute "good" halfmers. This liberal interpretation of the data implies that if "bad" halfmers were eluted by the smaller amount of guanidine-HCl, half of them must have subsequently become "good" halfmers when dialyzed into Tris buffer. Proof of this interpretation would substantiate the theory of conformational differences between opposing binding sites of a "7S" molecule. 2) The more conservative interpretation (and more likely) of the data would be that the guanidine-HCl elution was not preferential but rather nonspecifically eluted 50% of the antibody. All but one of the other dissociating agents tried either eluted all of the antibody from the column or was not able to elute any of the antibody. Only recently it was observed that .5 M Tris-HCl (pH 8.5) could elute 50% of the "7S" antibody absorbed to the DNP affinity column. The use of .5 M Tris-HCl was prompted by an observation which warrants some discussion at this point. Rather serendipitously, it was observed that mild reduction in .5 M Tris-HCl of either of the two 19S molecules at dilute protein concentrations

(<1 mg/ml) yielded subunits of which 50% would bind to a DNP affinity matrix (active) and 50% would not (inactive) providing the protein was left undiluted as applied to the affinity column. If the reduced mixture was first concentrated ∿90% of the antibody would bind to the column. When examined by equilibrium dialysis the unconcentrated inactive fraction displayed no binding sites, whereas the active fraction displayed an average of one site per presumed 2H-2L chain 7S subunit (Figure 10). Interestingly, if the inactive fraction was first concentrated to > 1 mg/ml a small percentage (.3 sites/2H-2L chain subunit) of high affinity sites could be generated. SDS-acrylamide gel electrophoresis of each fraction demonstrated that these molecules were predominantly halfmers (H-L) in dissociating buffer (Figure 11).

Hapten Binding by Tryptic Fragments

The finding of only one active hapten binding site in each reductive subunit from the two hybridoma-derived IgM antibodies prompted studies aimed at separating "good" (hapten binding) from "bad" (no hapten binding) Fab fragments. The strategy employed for this purpose involved attempts at preparing such Fab's by trypsinizing reductive subunits. Originally each reductive molecule was subjected to trypsinization for various times and aliquots were analyzed by SDS-PAGE (Figure 12). It was observed that although relatively little digestion occurred within the first two hours, digestion to Fabμ's (defined as ∿50,000

Figure 10. Equilibrium dialysis using DNP-ε-aminocaproate of NP3-17 Cl-20 "7S" active (●——●) and inactive (○——○) fractions resulting from mild reduction at dilute protein concentrations (< 1 mg/ml). The unfractionated starting material was identical to the active fraction. Concentration of the inactive fraction (⊗——⊗) resulted in the generation of some sites.

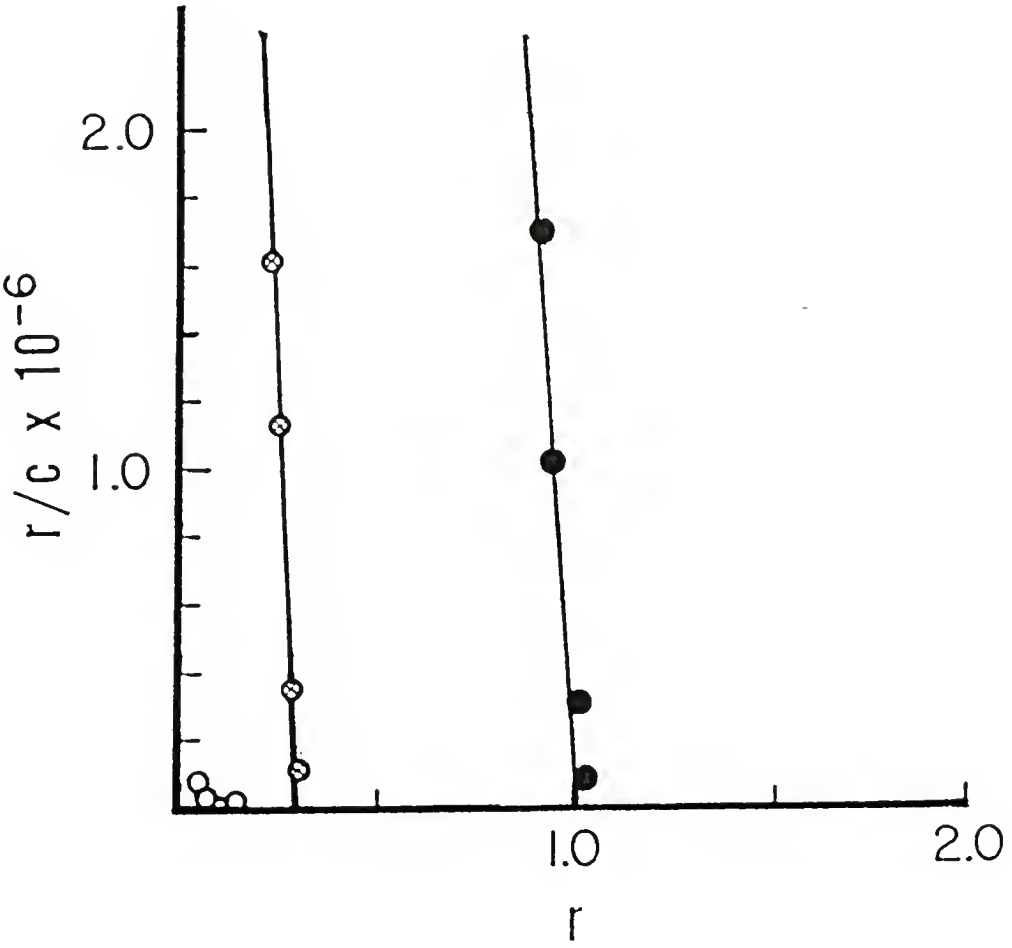

2.0
1.0
r/c x 10⁻⁶
1.0
2.0
r

Figure 11. 10% polyacrylamide gel electrophoresis (SDS) of NP3-17 C1-20 "7S". Lane A, unfractionated; Lane B, active fraction, and Lane C, inactive fraction. D, E, and F depict the SDS-PAGE patterns of samples A, B, and C after extensive reduction.

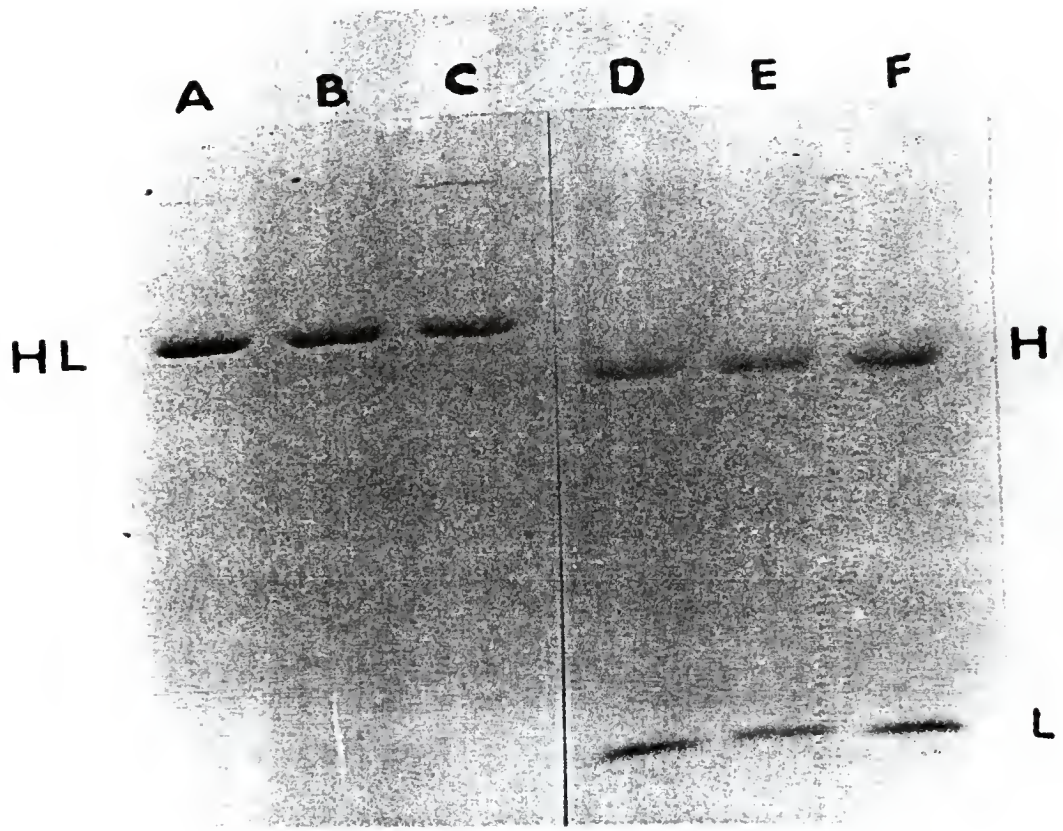
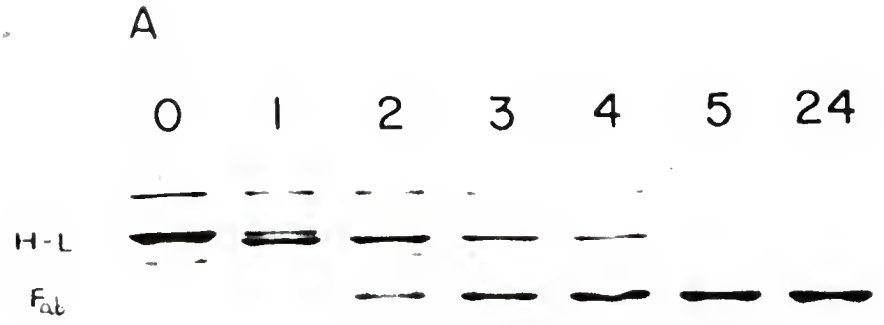
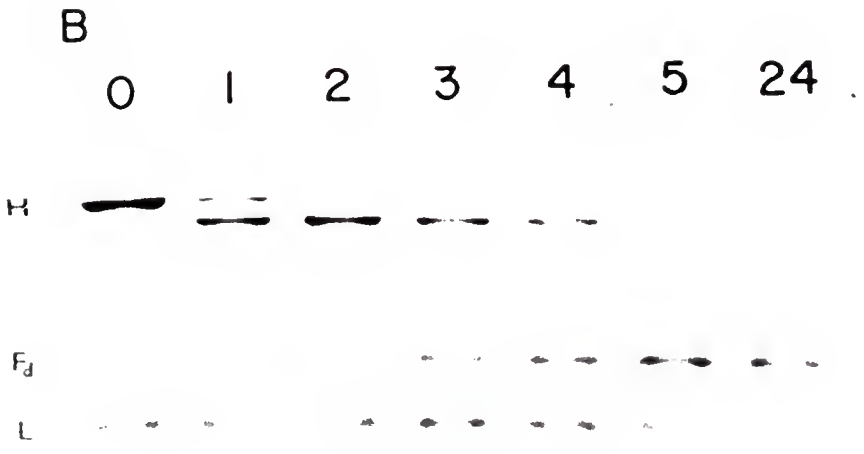

A
B
C
D
E
F
HL
H
L

Figure 12. 10% polyacrylamide gel electrophoresis of timed trypsin digestions of SP2/0 I-64 C1-12 7S reductive subunits. Length of exposure to trypsin is designated in hours above each sample. Panel A depicts unreduced samples; Panel B depicts the same samples upon reduction with .1 M 2 ME in 1% SDS in a boiling H_2O bath for three minutes.


A
0
1
2
3
4
5
24
H-L
Fab


B
0
1
2
3
4
5
24
H
Fd
L

dalton material containing L chains and part of the μ chains) was fairly rapid between 3-5 hours; very little change was seen over the course of the subsequent 19 hours. In addition, no $F(ab)_{2\mu}$'s were obtained since the inter-H chain disulfides were cleaved by the mild reduction step used in preparing the subunits. Large scale digestions were performed for periods longer than three hours (five hours for SP2/0 I-64 C1-12 and 18 hours for NP3-17 C1-20). Very little difference, if any, could be detected in their digestion profiles (G-200 and SDS-PAGE). The results of these large scale digestions are represented in Figures 13 and 14. Surprisingly, only 30-35% of the total protein (∿50% of the expected yield) was recovered as Fab's when the digested material was chromatographed on G-200 Sephadex equilibrated in Tris buffer. The remainder of the material was seen as small peptides. However, as demonstrated in Figure 15, the Fab's which were recovered displayed one high affinity binding site as measured by equilibrium dialysis. An important finding was that those Fab's containing one site accounted for ∿90% of the binding sites present in the untrypsinized starting material. Thus the digested Fab's seemingly were exclusively of the type that contained no active sites for binding the DNP ligand.

Asymmetry of Carbohydrate

One possible explanation for heterogeneity of binding within the pentamer and reductive subunits of these molecules could be the presence of an asymmetrical attachment of

Figure 13. Sephadex G-200 elution profile of SP2/0 I-64 C1-12 Fab's obtained after trypsinization for five hours. An eighteen hour trypsinization of NP3-17 C1-20 produced a similar profile.

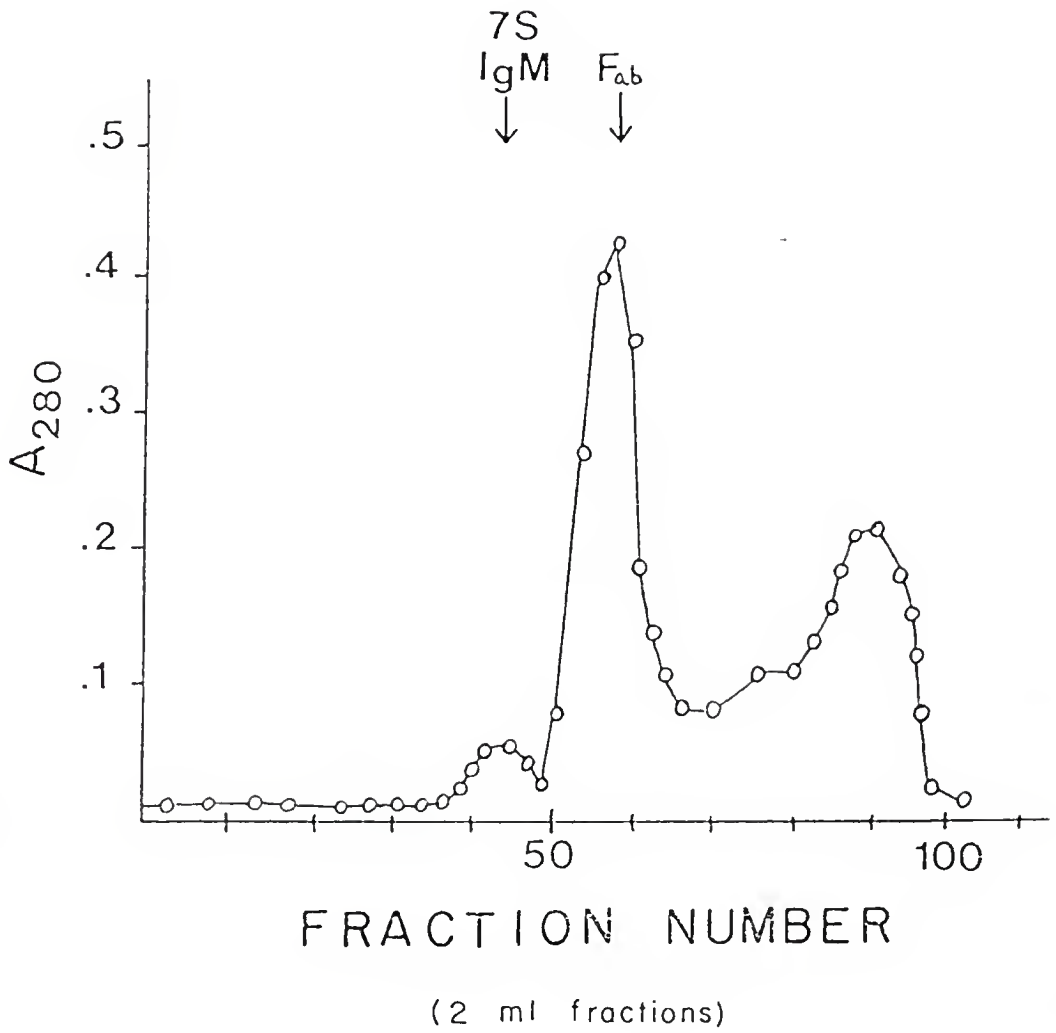

7S
IgM
Fab
.5
.4
.3
.2
.1
A280
50
100
FRACTION NUMBER
(2 ml fractions)

Figure 14. 10% polyacrylamide gel electrophoresis (SDS) of unfractionated (A), and active (C) Fab's obtained from five hour trypsinization of SP2/0 I-64 C1-12; (B) contains inactive, non-Fab material. D, E, and F depict the SDS-PAGE patterns of samples A, B, and C after extensive reduction. SDS-PAGE patterns of NP3-17 C1-20 Fab's were indistinguishable.

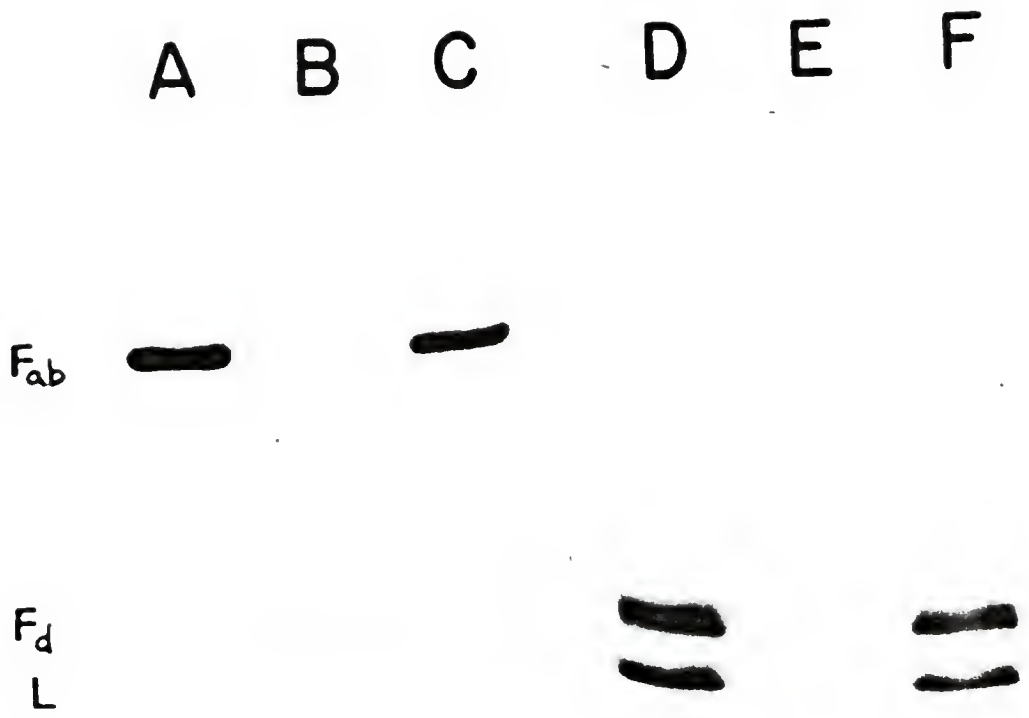
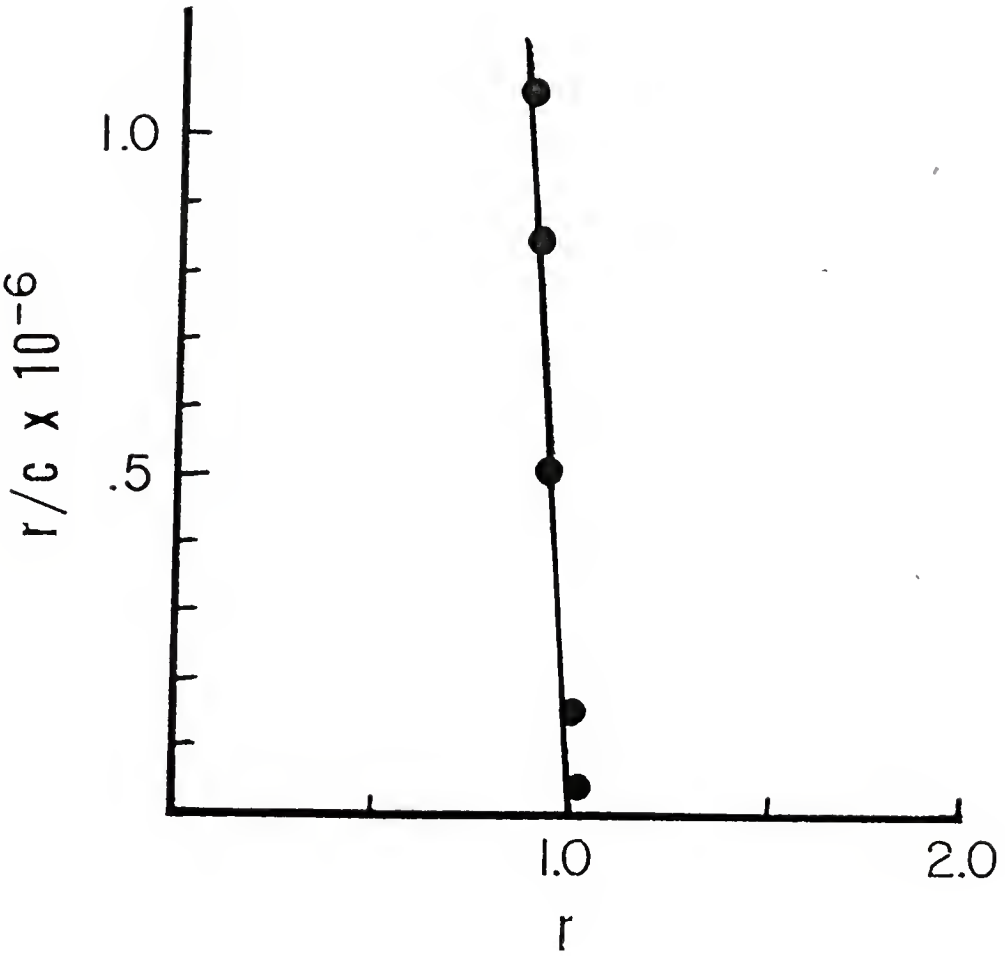

A
B
C
D
E
F
Fab
Fd
L

Figure 15. Equilibrium dialysis of Fab fragments obtained from a five hour trypsinization of SP2/0 I-64 C1-12 with DNP-ε-aminocaproate. Results from equilibrium dialysis with Fab's from NP3-17 C1-20 were identical.


1.0
.5
r/c x 10⁻⁶
1.0
2.0
r

carbohydrate (see 47). This possibility prompted an experiment designed to obtain carbohydrate-free IgM antibodies. Tunicamycin (Tm), an antibiotic that prevents glycosylation of glycoproteins by blocking the formation of M-acetyl-glucosamine-lipid intermediates (48), was used to block glycosylation of the IgM produced by SP2/0 I-64 C1-12. It was found that culture supernatants from ^{35}S-methionine-labeled cells treated for eight hours with Tm contained only 1% (15,000 cpm) of the antibody level (1.5 x 10^6 cpm) found in supernatants from non-Tm treated ^{35}S-methionine labeled cells. Furthermore, the IgM that was purified from the supernatants of Tm-treated cells appeared to be no different from IgM purified from the supernatants of non-Tm treated cells. This was evident by an autoradiogram of purified antibody run on an SDS-DATD acrylamide gel (Figure 16) and by sequential hapten elutions of reductive 7S subunits (Table 3). Radioactive material which was not specifically purified by an affinity column was shown by immunoprecipitation to be free of IgM (Table 4). Attempts were made to isolate carbohydrate-free antibody from cell lysates of Tm-treated cells, but unfortunately, none was detected. It is not known if lower levels of Tm or other incubation times might be advantageous for the production of unglycosylated IgM by this cell line.

Anti-idiotypic Antiserum

In terms of assessing the variability of antibody sites, the utilization of anti-idiotypic antibodies has become a

Figure 16. Autoradiogram of specifically purified ^{35}S-methionine molecules from supernatants or cell lysates of Tm- or non-Tm-treated SP2/0 I-64 Cl-12 cells.
Lane A and H - Purified antibody from non-Tm-treated cultures;
Lane B and G - Purified antibody from Tm-treated cultures;
Lane C and F - Purified antibody from non-Tm-treated cell lysates;
Lane D and E - Purified antibody from Tm-treated cell lysates.
Samples E - H were reduced with .1 M 2 ME and boiled in 1% SDS for three minutes.

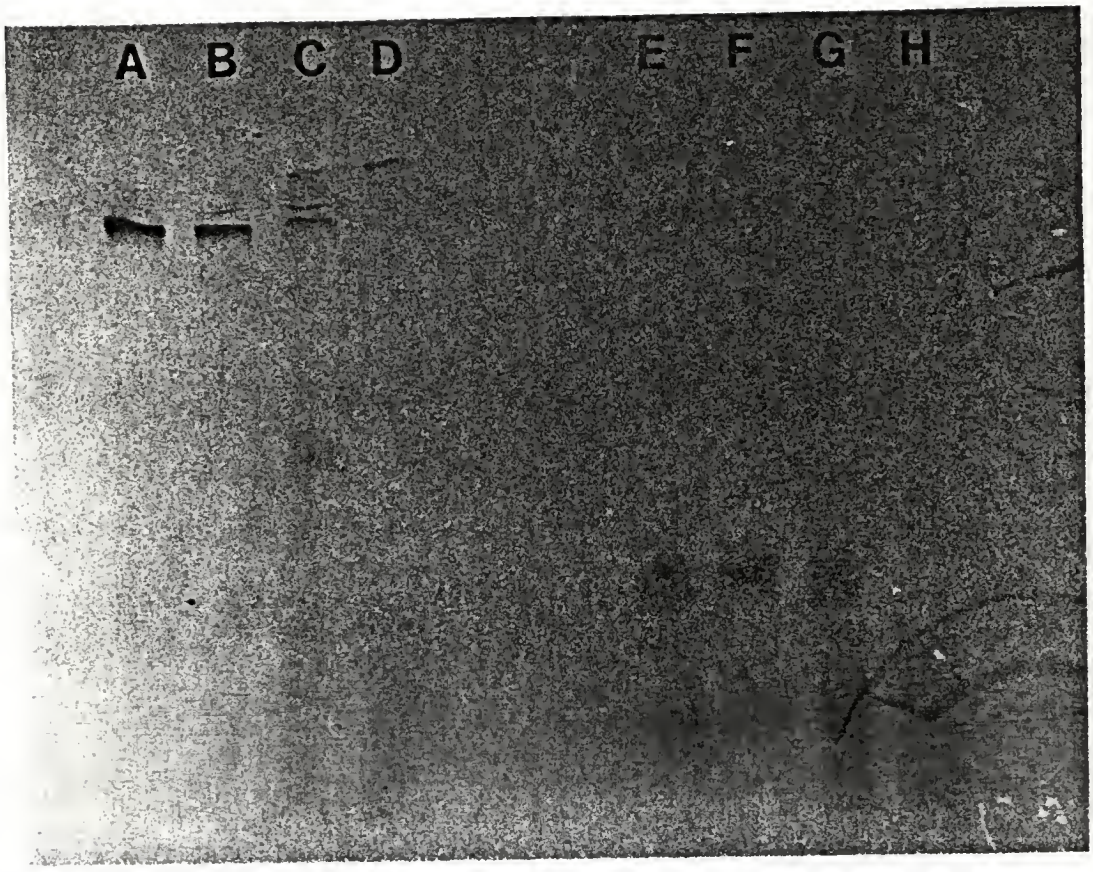

A
B
C
D
E
F
G
H

TABLE 3

Comparison of Hapten Elution Profiles of
Mildly Reduced SP2/0 I-64 C1-12 IgM Antibody
from Tm- and Non-Tm-treated Culture Supernatants

(DNP-OH) Hapten Concentration	Antibody from Non-Tm-Treated Culture Supernatants[1]			Antibody from Tm-Treated Culture Supernatants[2]		
	CPM Eluted		OD_{280} Eluted[3]	CPM Eluted		OD_{280} Eluted
	(% of Total)			(% of Total)		
5×10^{-5} M	0		0	0		0
1×10^{-4} M	4200	(10.5)	.275	650	(8.0)	.210
5×10^{-4} M	7500	(18.8)	.267	1000	(12.5)	.270
1×10^{-3} M	17000	(43.0)	.328	3600	(45.0)	.342
5×10^{-3} M	4800	(12.0)	.264	650	(8.0)	.180
1×10^{-2} M	0		0	0		0
	34000	(85.0)	1.1	5800	(73.0)	1.002
Inactive Fraction	4000	(10.0)	.2	1500	(19.0)	.18
Total	38000	(95.0)	1.3	7300	(94.0)	1.182

[1] Loaded 1 ml DNP-lys-sepharose affinity column with 40,000 cpm.

[2] Loaded 1 ml DNP-lys-sepharose affinity column with 8,000 cpm.

[3] .5 ml of non-radioactive SP2/0 I-64 C1-12 antibody ($O.D._{280}$ = 2.0) was added to each radioactive antibody preparation prior to reduction for use as an optical density marker.

TABLE 4

Immunoprecipitation of Tunicamycin (Tm)-treated and Non-Tm-treated Culture Supernatants and Cell Lysates

Sample	Amount of Rabbit anti-mouse μ chain (ul)	% of Radioactivity Precipitated
I. Control Antibody[A]	20	100[B]
	40	100
	100	100
	0 (100 ul Normal Rabbit Serum)	2
II. Culture medium spiked with Control Antibody	100	100
III. Inactive[C] fraction from non-Tm-treated cultures	100	.004
IV. Inactive fraction from non-Tm-treated culture supernatants	0 (100 ul Normal Rabbit Serum)	.008
V. Inactive fraction from Tm-treated culture supernatants	100	.004
VI. Inactive fraction from non-Tm-treated cell lysates	100	.005
VII. Inactive fraction from Tm-treated cell lysates	100	.004

A - ^{35}S-labeled affinity purified antibody from non-Tm-treated culture supernatants.

B - Percentages were normalized to 100% to account for a uniform loss of radioactive material throughout the experiment due to non-specific stickiness.

C - Radioactivity which was not absorbed by the DNP-lys-sepharose affinity column (i.e. antibody to DNP removed).

powerful tool (49). The purpose of developing such an antiserum in this study was hopefully to allow detection of idiotypic differences between "good" and "bad" halfmers. Unfortunately no anti-idiotypic antibodies could be detected in the ascitic fluids of these animals by Ouchterlony analysis against the intact 19S molecule. Perhaps a better approach might be to immunize guinea pigs and absorb this antiserum with the necessary mouse proteins until desired idiotypic specificity is achieved.

Affinity Labeling

Affinity labeling of various anti-DNP antibodies has been successfully utilized to either completely or partially block the antigen combining site via covalently coupling antigen in the site (50). This approach was attempted with hopes of obtaining "7S" subunits with inactive sites. By placing these inactive subunits in 2.0 M guanidine-HCl and subsequent dialysis against Tris-buffered saline the generation of active sites might be possible. This result would argue that inactive sites have the capability of binding to antigen but when paired with an active site assume the wrong conformation for antigen binding. Unfortunately in this experiment conditions were not appropriate for affinity labeling as no inactive antibody passed through the affinity column. It is quite possible that the appropriate conditions for affinity labeling could be achieved and should be pursued in future studies.

Discussion

The purpose of the work described in Chapter III was to utilize murine hybridomas as sources of homogeneous IgM antibodies to test the hypothesis that certain IgM antibodies can exhibit intramolecular heterogeneity of ligand binding that is not attributable to primary structural differences. The results of polyethylene glycol induced fusions using non-immunoglobulin producing cell lines indicated that stable, antigen specific, IgM secreting cell lines can be obtained. The two IgM antibodies obtained from the hybridomas displayed an unusually high affinity ($\sim 10^7 M^{-1}$) for the DNP hapten. It is not known if cells producing lower affinity IgM antibodies (10^4-$10^5 M^{-1}$) were present as a result of the fusion process and were missed by the screening procedure used or if, for one reason or another, cells producing such antibodies simply did not exist. Nevertheless, these antibodies did meet the necessary criteria for testing the original hypothesis in that 1) they were specifically induced pentameric antibodies which displayed binding heterogeneity, i.e., only five DNP binding sites. This heterogeneity appeared to be of an intramolecular nature as opposed to intermolecular heterogeneity based upon the following premises. It seems rather unlikely, due to the nature of hybridoma derived antibodies, that the two hybridoma cell lines derived (NP3-17 Cl-20 and SP2/0 I-64 Cl-12) from the two different myeloma cell lines used (NP3 and SP2/0) could both manufacture and secrete

in equal amounts two nondistinct populations of IgM antibodies, one of which had ten binding sites for DNP and one of which had no binding sites for DNP. Furthermore, essentially all (> 90%) of the "7S" reductive subunits absorbed to (and subsequently hapten eluted from) a DNP-lys-sepharose column. These subunits when measured by equilibrium dialysis displayed an average of one site per 7S subunit. These results clearly support the interpretation that each 7S subunit must contain only one site, i.e., there were none which contained two and likewise there were none which displayed no DNP-binding sites. 2) These IgM antibodies also met the criterion of being structurally homogeneous, at least by the methods employed. Comparison of alkaline urea gel banding patterns of myeloma protein L chains with the banding patterns of these hybridoma L chains demonstrated considerable structural homogeneity (for example, compare with the banding pattern of MOPC-21 L chain, Figure 4, Chapter II). Likewise limited amino acid sequence analysis certainly indicated homogeneity with NP3-17 C1-20. Undoubtedly, complete amino acid sequences of both proteins or sequence analysis at least through the first hypervariable region would be invaluable in this regard. However, the question becomes more pertinent when putative inactive sites are separated from active sites. Perhaps at that stage additional sequence data, as well as peptide mapping, isoelectric focusing, and/or idiotypic analysis would be beneficial.

The results from mild reduction of both IgM antibodies indicated that the 19S molecules proceeded directly from the 19S covalent pentameric form to covalent halfmers (H-L). For example Figure 7 demonstrates that a concentration of .001 M 2ME does relatively little to change the covalent structure of the molecule whereas a concentration of .005 M 2ME has converted all of the heavy molecular weight molecules predominantly to halfmers. The obvious interpretation of this finding is that for these molecules the inter-heavy chain disulfides are as susceptible to reduction as are the inter-subunit disulfides. This fact could become important in uncovering a possible mechanism for intramolecular heterogeneity. According to Askonas and Parkhouse (51, p. 632) the intracellular assembly pathway for IgM "mirrors the resistance of interchain disulphide bonds to reduction." If this is true with these hybridoma-derived molecules, it would appear that these molecules are assembled through a H-L chain intermediate based upon their reduction profiles. If assembly of IgM is involved in the observed heterogeneity, comparisons of reduction profiles of these IgM antibodies with reduction profiles of other IgM antibodies which possess ten homogeneous binding sites might bring to light an assembly intermediate (for example H-H-L, see 40) responsible for the phenomenon.

Examination of the results from experiments designed to separate noncovalently associated halfmers yielded few clear interpretations. This was in part due to the fact that the

amount of dissociating agent used (two column volumes of 2.0 M guanidine-HCl) was critical in removing 50% of the antibody absorbed on the affinity column. Any more than this amount or subsequent elutions with the same amount eluted more antibody from the column. As pointed out previously the most likely interpretation of these results would be that the guanidine-HCl elution was not preferential but rather nonspecifically eluted 50% of the antibody. Likewise, results obtained when .5 M Tris-HCl was used to dissociate the halfmers were equally difficult to interpret due to the problems associated with total recovery of hapten binding sites. Neither the 50% fraction which was eluted from the affinity column by .5 M Tris-HCl nor the 50% fraction which passed through the DNP-immunoabsorbant when the mild reduction was performed on dilute solutions of antibody, displayed any active binding sites by equilibrium dialysis. The hapten eluted fractions (the remaining 50%) in both cases displayed only one active site per 2H-2L chain subunit, thus accounting for only one half of the original sites in the unfractionated subunit population. Although equilibrium constants were not measured for the noncovalent association of halfmers used in this study, one published report of halfmer association using three other IgM monoclonal antibodies gives an equilibrium constant of 2.3×10^{-6} moles halfmers^{-1} for the reaction one halfmer $\rightleftharpoons$ two halfmers (52). It is quite possible that equilibrium constants of this magnitude or higher may exist for

the hybridoma-derived IgM antibodies described in this study. If so, separation of the putative "good and bad halfmers" using chaotropic or dissociating agents may be impossible, as these agents may equally disrupt hapten binding and halfmer association. Unlike the previous study mentioned on halfmer association Parkhouse (53, p. 640) in describing halfmer association with IgM from the mouse myeloma MOPC 104E states:

> Since, therefore, there is no pronounced tendency for non-covalent interaction between HL subunits it is no surprise to find that the equilibrium for the reaction 2HL→IgMs (7S) is not completely in favour of IgMs.

Quite possibly, intramolecular heterogeneity may arise not as a result of the mode of assembly of the IgM molecule but rather by the conformation that must be assumed by one halfmer as it associates with another halfmer for which it possesses strong noncovalent attractions. Likewise, those molecules lacking strong noncovalent attractions can readily form two functional binding sites per 2H-2L chain subunit. Future experimentation in this area could provide some rewarding information about intramolecular heterogeneity.

The most exciting observation concerning the possibility of conformational differences between opposing binding sites of the same 7S subunit was seen when tryptic fragments were obtained from both IgM antibodies. As demonstrated in Figure 13 only 50% of the expected yield of Fab$_\mu$'s was obtained by gel filtration on G-200 Sephadex. Approx-

imately 90% of these Fabμ's absorbed to and were subsequently hapten eluted from a DNP-lys-sepharose affinity column. Each of these Fab's contained one site as measured by equilibrium dialysis (Figure 15) and accounted for ∿ 90% of the binding sites present in the untrypsinized starting material. The obvious conclusion from these results is that the Fab's which were digested and therefore not recoverable were exclusively of the type that contained no active sites for binding the DNP ligand. This finding is best explained by the presence of two populations of conformationally distinct Fab's. It is hoped through future experimentation with trypsin, or one of the other available proteolytic enzymes, that conditions which will allow for near 100% recovery of the Fab's can be ascertained. Accomplishment of such a task will allow for careful investigation at both the structural and functional levels of each population of Fab's.

The finding of trypsin-sensitive Fab's might have some connection with an observation made while attempting to separate the two putative (good and bad) halfmers. The 50% fraction which passed through the affinity column after reduction of the IgM in dilute solution was found to be extremely labile. Immediate analysis of this fraction after affinity chromatography demonstrated the fraction was predominantly (>90%) halfmers (Figure 11). However, storage at 4°C overnight or freezing and thawing of this fraction produced a protein solution of which only a small

percentage was able to be concentrated using negative pressure dialysis. The remainder of material passed through the dialysis bag (MW exclusion of 12,000-15,000). Similar treatment of the 50% hapten eluted fraction had little effect. It is conceivable that this labile halfmer fraction, like the trypsin-sensitive Fab's, had obtained a conformation which when purified in dilute solution was extremely sensitive to proteolysis.

Recent studies have shown that the structure of the carbohydrate on the H chain of certain antibodies may influence the strength of the interaction of those antibodies with polymeric antigen (54). Studies suggesting the importance of the protein structure in determining glycosylation have also been reported (55,56). These studies in conjunction with one report of asymmetrical attachment of carbohydrate to rabbit IgG prompted the experiments in this study with the antibiotic tunicamycin. One possible explanation of two distinct conformations for a single protein could be the presence or absence of carbohydrate. Unfortunately, as predicted by previous studies with Tm (57), secretion of IgM from these cell lines was dramatically decreased ($\sim$ 99%) in the presence of Tm. Likewise under these experimental conditions no intracellular IgM from Tm-treated cells could be isolated. Perhaps future endeavors with this antibiotic may determine a suitable concentration and incubation period which would

allow for secretion of unglycosylated IgM. One possible alternative to this approach might be mechanical stripping of purified IgM.

CHAPTER IV
SUMMARY AND CONCLUDING REMARKS

The objective of this research was to clearly resolve the question of intramolecular heterogeneity of ligand binding by homogeneous IgM antibodies. This has been an area of controversy for over a decade with relatively few advancements being made in the last five years. By utilizing hybridoma-derived IgM antibodies many of the problems associated with this unresolved question have been alieviated. This work also attempted to determine if intramolecular ligand binding differences are due to primary structural differences or conformational differences.

Chapter II described a somewhat unusual IgM molecule, 14PAF. This 19S IgM antibody exhibited an average of six homogeneous relatively high affinity binding sites per molecule for the DNP moiety. The failure to demonstrate ten binding sites per pentameric molecule was attributable to the presence of two different light (L) chains in the secreted molecules. One of the L chains appeared, based upon limited amino acid sequence studies, to be the L chains normally found in MOPC-21, the myeloma protein secreted by the P3 myeloma line. The other L chains, presumably derived from the murine spleen cell used in the fusion, were essential for anti-DNP combining sites. Analysis of the reductive subunits of the secreted IgM

anti-DNP molecules indicated the presence of two types. One type (designated active) absorbed to DNP affinity matrices, contained an average of two homogeneous ligand binding sites per subunit and did not contain MOPC-21 L chains. The other type of subunit (designated inactive) did not contain any DNP binding sites and contained only MOPC-21 L chains.

Polypeptide chain recombination studies with mildly reduced and alkylated μ and L chains from each type of reductive subunit indicated that the μ chains were functionally identical. Furthermore these in vitro recombination studies indicated that the noncovalent assembly of the 2μ-2L chain subunits was restricted by the L chains in such a way that the recombinant subunits were homogeneous in terms of L chains. Equilibrium dialysis studies with active homogeneous recombinant subunits indicated the presence of but one ligand binding site per 2μ-2L chain subunit.

Chapter III described the structural and functional analysis of two additional murine anti-DNP IgM hybridoma antibodies, NP3-17 C1-20 and SP2/0 I-64 C1-12. Each of these 19S IgM molecules displayed an average of five high affinity binding sites for the DNP moiety. Furthermore, ≥ 95% of the reductive subunits of each of these proteins absorbed to and were hapten eluted from a DNP-lys-sepharose affinity column. These subunits, when examined by equilibrium dialysis using DNP-ε-aminocaproate, indicate each to

contain an average of one high affinity binding site. Structural analysis of each molecule indicated each to be homogeneous. The most surprising finding with these molecules was the ability to recover only 50% of Fab's following trypsin hydrolysis. Interestingly, the 50% that were recovered accounted for all of the binding sites present in the starting 7S material. This finding, as well as limited success with the separation of inactive reductive halfmers (H-L) which subsequently became active, indicate that differences in conformation of the binding sites may account for the observed binding heterogeneity. Although the study with 14PAF (Chapter II) did not directly answer any questions regarding intramolecular heterogeneity, the results of the chain recombination studies (one ligand binding site per 2H-2L chain recombinant subunit) closely mimic the natural situation that occurs with the two molecules discussed in Chapter III, i.e. one binding site per 2H-2L chain subunit. These findings strongly implicate the mechanism of assembly as a possible factor in the generation of conformational differences that could account for the intramolecular heterogeneity of ligand binding seen with certain IgM molecules.

It is hoped that these studies have laid a foundation from which important questions regarding the structure, function, and intracellular assembly of IgM antibodies may be answered, as well as uncovering other potentially rewarding areas of research. Optimistically, extensions of these

studies may result in a wide variety of new approaches to probing and understanding B cell activation and regulation.

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BIOGRAPHICAL SKETCH

The author was born December 15, 1952, in Tupelo, Mississippi, as the third child of Rex and Gertrude Giles. Bob graduated from Tupelo High School in 1970, and attended Mississippi State University from 1970-1974 on scholarship as an athletic trainer for the Bulldogs. After receiving a Bachelor of Science degree in general science with a major in zoology and a minor in chemistry, Bob enrolled in the master's program in the Department of Microbiology at Mississippi State to study under Dr. Lewis R. Brown. It was also at this time that he was married to Jansen Lamberson. The author received his Master of Science degree from Mississippi State in 1976, and remained in the Microbiology Department for approximately one year as a Research Associate. He enrolled at the University of Florida in the Fall of 1977 in order to obtain his Doctor of Philosophy degree in immunology. After acceptance into candidacy for this degree Bob elected to follow his mentor, Dr. L. William Clem, to the University of Mississippi Medical Center, Jackson, Mississippi, where Dr. Clem had recently been appointed as Chairman of the Department of Microbiology. While in Jackson, Bob and Jansen were blessed by the birth of their son Robert Clay, Jr. After graduation from the University of Florida the author plans to do post-doctoral work in the laboratory of Dr. J. Donald Capra at the University of Texas Health Science Center at Dallas, Dallas, Texas.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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